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# INVESTIGATION OF THE ROLES OF ASF1 AND CAF-1-MEDIATED CHROMATIN ASSEMBLY IN THE HUMAN DNA DAMAGE RESPONSE

Ting-Hsiang Huang

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INVESTIGATION OF THE ROLES OF ASF1 AND CAF-1-MEDIATED  
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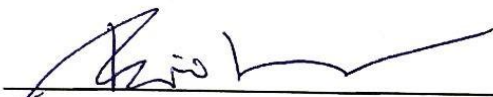
by

Ting-Hsiang Huang, M.S.

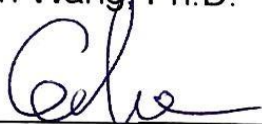
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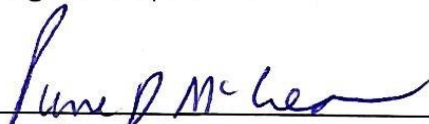
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INVESTIGATION OF THE ROLES OF ASF1 AND CAF-1-MEDIATED  
CHROMATIN ASSEMBLY IN THE HUMAN DNA DAMAGE RESPONSE

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Ting-Hsiang Huang, M.S.

Houston, Texas

May, 2017

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## Dedication

*This dissertation is dedicated to my beloved life teacher JMS for his guidance and my wife, Tanya, for her unconditional love, continuous encouragement and support as well as my parents for their sacrifice, without which it would have been possible for me to make this achievement.*

## Acknowledgements

I would like to first thank my mentor Dr. Jessica Tyler for being such a tremendous scientist role model in my research path. Without her support in many aspects, encouragement and trust, I would not been able to grow professionally and scientific-wisely think outside of the box. I would also like to thank all of my committee members, Drs. Bin Wang, Grzegorz Ira, Pierre McCrea, Xiaobing Shi, Hui-Kuan Lin and Randy Legerski for lending their expertise to this work. I would express my acknowledgement to Dr. Barry Sleckman for his scientific input on the project by which this work was raised to a higher level.

For people who have ever participated throughout the course of my Ph.D. studies, I am eternally grateful for your support which is very critical to my success.

Surviving from PhD loneliness and solitude is definitely a mental training process of being mature individuals. Matthew 17:20 says, “Because you have so little faith. Truly, I tell you, if you have faith as small as a mustard seed, you can say to this mountain, ‘Move from here to there’ and it will move. Nothing will be impossible for you.” I realized, with faith as a mustard seed inside my heart, now a mustard tree has grown up from the seed.

*Lastly but most importantly, thank the Holy Trinity and SSN for everything you have  
done for me!*

# INVESTIGATION OF THE ROLES OF ASF1 AND CAF-1-MEDIATED CHROMATIN ASSEMBLY IN THE HUMAN DNA DAMAGE RESPONSE

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Advisory Professor: Jessica K. Tyler, Ph.D.

The access-repair-restore model for the role of chromatin in DNA repair infers that chromatin is a mere obstacle to DNA repair. However, here we show that blocking chromatin assembly of newly-synthesized histones, via knockdown of the histone chaperones ASF1A, CAF-1 or a mutation that specifically prevents ASF1 binding to histones, hinders loading of Rad51 onto ssDNA during homologous recombination, as a consequence of reduced recruitment of the Rad51 loader MMS22L/TONSL to ssDNA, resulting in persistent RPA foci, extensive DNA end-resection, and persistent activation of the ATR-Chk1 pathway. By contrast, ASF1 and CAF-1 render the rapid inactivation of ATM-Chk2 pathway via facilitating histone acetylation to allow the recruitment of ATM to DSBs for efficient repair by non homologous end joining. Furthermore, in response to DSB damage, ASF1A is specifically phosphorylated by DNA-PKcs and potentially other kinases, enhancing its interaction with histones H3.1/H4 and CAF-1, and promoting the recruitment of MMS22L/TONSL to the damaged DNA to form the Rad51 nucleofilament during homologous recombination. We propose a model whereby DSB-induced phosphorylation of ASF1A promotes transient assembly of newly-synthesized histones onto single-stranded resected DNA, which serves to recruit TONSL-MMS22L to efficiently form the Rad51

nucleofilament for strand invasion. As such, we have uncovered an unexpected role for chromatin assembly in an intermediate stage of homologous recombination, revealing that chromatin assembly plays an active role in DNA repair *per se*.



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# **Chapter 1**

## **Introduction**

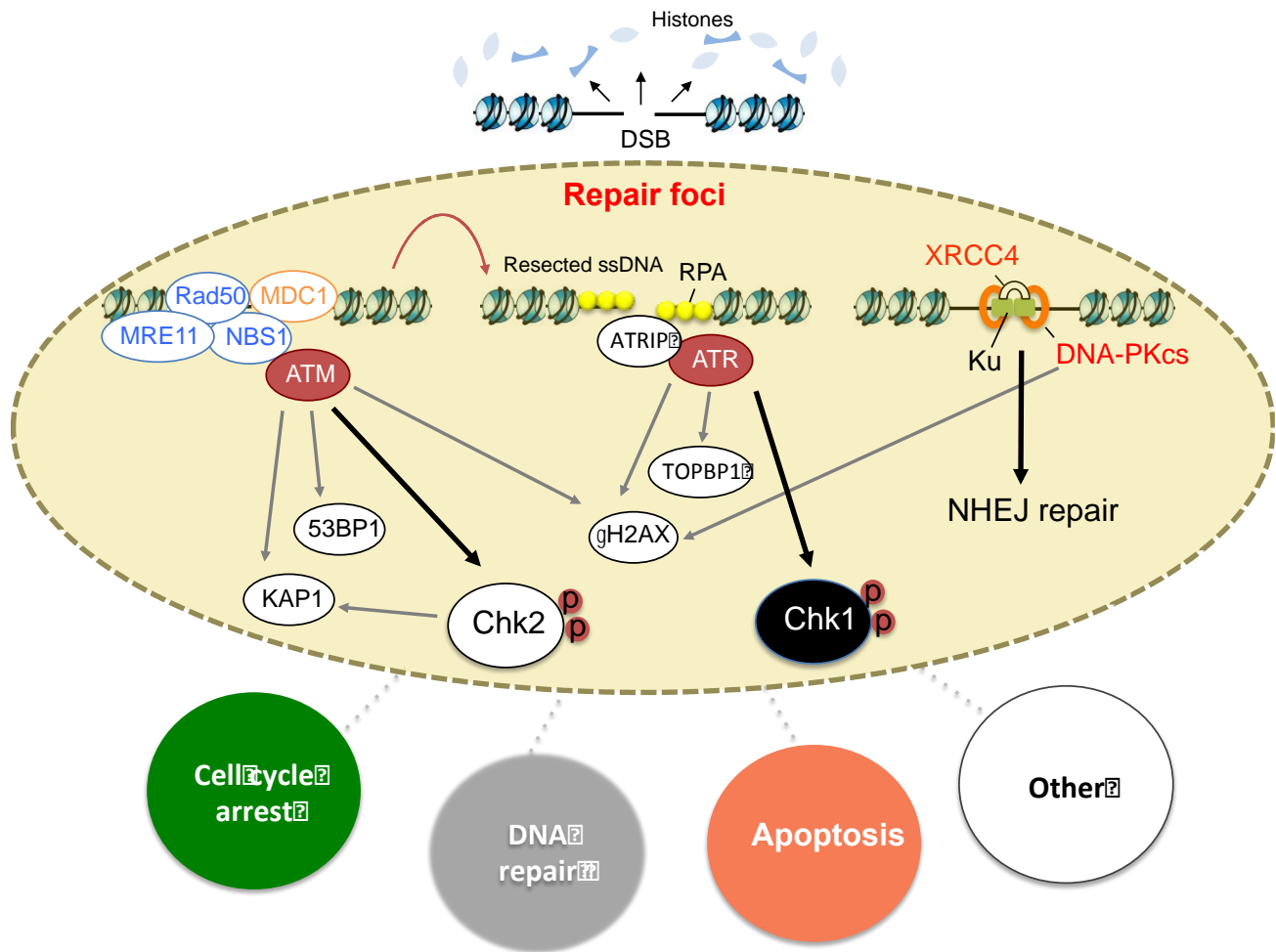


## 1.1. The DNA Damage response

The inability of cells to accurately perform DNA repair in response to DNA insults causes cells to become genetically unstable and predisposed to human diseases including cancer. Cells have evolutionarily developed rapid and efficient cellular DNA-repair mechanisms, termed DNA damage responses (DDR) to sense DNA lesions, spread signals and coordinate the control of cell cycle with repair processes to maintain genomic integrity. The DDR is an entangled network which is composed of various components typically classified into four categories including DNA damage sensors, mediators, signal transducers and effectors (Sancar et al., 2004) (**Fig. 1**). The fundamental core element for cells to accurately conduct complicated DDRs relies on protein-protein interactions giving rise to proteomic post-translational modifications including phosphorylation, ubiquitination as well as SUMOylation and protein translocations such as recruitment and displacement. When cells encounter genomic insults, sensors are responsible for recognizing DNA lesions and structural alterations in the genome (Harper and Elledge, 2007). In mammalian cells, Mre11-Rad50-Nbs1 (MRN) complex serves as a sensor that rapidly accumulates at DNA double-stranded break (DSB) sites (Uziel et al., 2003), allowing further DNA end processing and recruitment of DNA damage mediators to selectively coordinate with signal transducers and effectors according to cell states. It is appreciated that one type of effector, called the DNA damage checkpoint proteins, while activated, would lead to cell cycle arrest that gives cells enough time to fix the DNA breaks and resume cell cycle progression until repair is completed. If DNA

lesions are not well resolved, cells are programmed to death, termed apoptosis, so as to prevent them from passing errors in genetic materials to daughter cells (Fig. 1).

**Figure 1**



**Figure 1. The canonical DNA damage response (DDR) pathways in mammalian cells. The DDR pathways are composed of numerous proteins to form a complicated network in which the signal transduction is mainly**

mediated through protein-protein interaction and post-translational modifications (such as phosphorylation, ubiquitination and SUMOylation etc...) from upstream kinases towards downstream cellular responses. DNA damage signaling cascade in mammals is typically regulated by ATM and ATR as well as DNA-PK depending on the type of DNA lesion. These three signaling pathways have distinct or substitutive functions to each other.

## **1.2. Phosphoinositide 3-Kinase-like Kinases (PIKKs)**

Phosphoinositide 3 kinase-like kinases are a serine/ threonine kinase family composed of six members including Ataxia-Telangiectasia Mutated (ATM), Ataxia-Telangiectasia and Rad3-related (ATR), DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs), mammalian Target of Rapamycin (mTOR), Suppressor of Morphogenesis in Genitalia (SMG1) and Transformaion/ Transcription Domain-Associated Protein (TRRAP) (Abraham, 2004). ATM and ATR as well as DNA-PKcs are regarded as core DNA damage mediators that facilitate protein-protein interactions and initiate signal transductions by phosphorylating sequential substrates.

### **1.2.1. Mechanisms of Ataxia-Telangiectasia Mutated (ATM) Activation**

Functional characteristics of ATM in maintaining mammalian genome stability was first noted in patients with a defect in the ATM gene, leading to severe developmental neurodegeneration disorders, cerebellar degeneration, immunodeficiency, chromosomal instability and incidence of cancer (Rothblum-

Oviatt et al., 2016). ATM protein exists as a dimer or a high order multimer in an inactive state (Bakkenist and Kastan, 2003). In the presence of DNA lesions, intermolecular autophosphorylation of ATM occurs at Serine 1981 site (ATM<sub>S1981P</sub>) causing the dissociation of the ATM dimer to monomers and localization to sites of DNA breaks (Bakkenist and Kastan, 2003). Monomeric ATM is recruited to DSB ends by the MRN complex (Lee and Paull, 2007). The epistatic link between ATM and the MRN complex arose from patients with genetic mutations in Mre11 or Nbs1 sharing common rare clinical syndromes (Ataxia-Telangiectasia-Like Disorder) with A-T patients (Taylor et al., 2004). Indeed, MRN knockout mice have embryonic lethality while MRN deficient cells showed a significant reduction in ATM activation that also affected phosphorylation of downstream factors such as Chk2 and p53 (Uziel et al., 2003). However, a recent study indicates ATM activation and its recruitment to chromatin still occur in the absence of MRN and the inhibition of DNA-PK, suggesting that even though MRN complex promotes ATM activation, it is not essential for ATM activation and perhaps helps amplify and sustain ATM activity in response to DSB (Hartlerode et al., 2015).

DNA end structure also affects ATM activation. ATM recognizes and favorably interacts with open and unwound DSB ends *in vivo* with the help of helicase activities possessed by RAD50 in the MRN complex and *in vitro* with dsDNA alone (Paull, 2015). A study showed long 3' or 5' single-stranded DNA overhangs inhibit both MRN and ATM binding in mammalian extracts but

potentiated the activation of ATR in a length-dependent manner, giving rise to a switch between two PIKK components, ATM and ATR (Shiotani and Zou, 2009).

On top of that, chromatin is regarded as a critical determinant to maintain efficient and sustained ATM activity. The nucleosome-binding protein HMGN1 has been found to bridge ATM and chromatin through the interaction with histone H3 lysine 14 acetylation (H3K14ac) which is increased in a HMGN1 dependent manner upon IR irradiation (Lim et al., 2005). Cells lacking HMGN1 have attenuated ATM activation and p53 phosphorylation whereas the addition of histone de-acetyltransferase (HDAC) inhibitor elevated phosphorylation levels of ATM regardless of HMGN1 during IR treatment, suggesting the involvement of other acetyltransferases in this process (Kim et al., 2009). Indeed, another non/histone acetyltransferase Tip60 that is associated with chromatin-bound histone H3 tri-methylated on lysine 9 (H3K9me3) has been reported earlier than HMGN1, to function in the activation of ATM by acetylating ATM on lysine 3016 in response to DNA damage (Sun et al., 2005; Sun et al., 2007). Moreover, histone H4 lysine 16 acetylation (H4K16ac) mediated by the MOF protein and SETD2-induced tri-methylation on H3 lysine 36 (H3K36me3) have been shown to promote efficient ATM activation (Carvalho et al., 2014; Sharma et al., 2010).

Taken together, the MRN complex is not the only factor to determine the activation of ATM, but instead, a growing amount of evidence provides a link between the context of chromatin and the initiation and / or duration of ATM activity in response to genomic insults. However, how chromatin structure transduces DNA damage towards ATM activity mechanistically is still elusive.

### **1.2.2. Mechanisms of Ataxia-Telangiectasia and Rad3-related (ATR) Activation**

Unlike ATM that is more responsive to DSBs, the ATR DDR mediator can be initiated and activated by various types of genomic insults, including DSBs, bulky adducts and especially SSBs generated from stalled replication fork or DNA end processing (**Fig. 1**). Although patients with mutations in ATR share common characteristics with ataxia-telangiectasia, called Seckel syndrome, most functions of ATM and ATR in DDR signaling pathways are distinct (Alderton et al., 2004).

Functional characteristics of ATR are mainly described during the replication-related DDR. When a replication fork stalls, single-stranded DNA (ssDNA) is generated on the lagging strand. In addition, different lengths of stretched ssDNA can be generated during nuclease-mediated DNA repairs, such as nucleotide-excision repair and homologous recombination (HR) following DNA damage. In order to protect vulnerable ssDNA from further nuclease attacks and forming secondary structures, replication protein A (RPA) rapidly binds exposed ssDNA, forming a platform where the ATR-interacting protein (ATRIP) anchors to recruit ATR to RPA-coated ssDNA (Ball et al., 2005). At dsDNA and ssDNA junctions, the Rad9-Hus1-Rad1 (9-1-1) complex forms a ring-shaped clamp and associates with Rad17 to co-regulate ATR activity (Parrilla-Castellar et al., 2004). Notably, this regulation requires Rad9 interaction with TopBP1 and the participation of Claspin for optimal ATR activation and phosphorylation of its substrates, for example, Chk1 (Kumagai et al., 2004). It has been shown that TopBP1 also interacts with ATRIP via its ATR-activating domain (AAD) and the TopBP1-

ATRIP interaction is strengthened by ATR-mediated TopBP1 phosphorylation, leading to a positive feedback loop in amplifying ATR signals (Yoo et al., 2007). This process is accompanied by complicated interplays between the above components upon various cellular circumstances.

Independently of DNA damage, DNA torsional stress caused by highly condensed chromatin during replication or chromatin structure alterations resulting from hyper- or hypo-tonic conditions induce a fraction of ATR activation and its redistribution to nuclear envelope (Kumar et al., 2014). When cells enter mitosis, ATR is required for chromatin condensation given that in the presence of ATR inhibitors or in Seckel cells (ATR deficiency) chromatin condensation is significantly delayed (Kumar et al., 2014). Intriguingly, a study indicated that site specific DNA damage-induced chromatin condensation following chromatin expansion triggers the initiation of DDR signaling, including ATM and ATR (Burgess et al., 2014). Both studies offer morphological evidence that chromatin dynamics affects ATR activity but the details of the mechanistic relationship requires further investigation.

### **1.2.3. Mechanisms of DNA-dependent Protein Kinase (DNA-PK) Activation**

DNA-PK consists of two components, the Ku heterodimer including two subunits Ku70 and Ku86 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and is primarily and majorly considered as a core non-homologous end joining (NHEJ) repair factor (**Fig. 1**)(Baumann and West, 1998). DNA-PKcs alone has a weak DNA binding activity (Yaneva et al., 1997) and therefore, requires Ku heterodimers as a prerequisite to be recruited to DNA regardless of

end structure but has a higher binding affinity to blunt end and overhangs in a sequence independent manner (Uematsu et al., 2007). Ku itself forms a ring structure binding to each side of DNA break ends that subsequently would allow one DNA-PKcs molecule to assemble at each end (DeFazio et al., 2002). The presence of activated DNA-PKcs at DNA ends ensures successful and efficient NHEJ repair. Functional DNA-PKcs relies on multiple phosphorylations in two important and conserved autophosphorylation clusters of DNA-PKcs (ABCDE and PQR) in vertebrates (Cui et al., 2005). Based on site mutation and functional analyses, those phosphorylation sites can be functionally redundant, reciprocal events, minimally contributing to or very important for DNA repair (Cui et al., 2005; Neal and Meek, 2011). The current model for DNA-PK activation will be described below.

In response to DNA lesions, DNA-PKcs molecules are recruited by Ku heterodimers to both of the opposing ends of DNA breaks in an unphosphorylated state followed by synapsis to tether two ends together and a subsequent series of autophosphorylations (Uematsu et al., 2007), but whether DNA-PKcs is activated *in cis* or *in trans* is not clear. It is believed that DNA-PKcs phosphorylation initially occurs within the ABCDE cluster, leading to a conformational change to allow accessibility to the ends of DNA (Ding et al., 2003). Interestingly, PQR cluster phosphorylation causes a subsequent conformational change to prevent DNA ends from further nuclease attacks but allows the process of DNA ligation (Cui et al., 2005). Moreover, the ablation of ABCDE or PQR cluster phosphorylation results in severe radiosensitive



phenotypes (Cui et al., 2005). In addition, the 7A mutant (S2056A, T2609A, S2612A, T2620A, S2624A, T2638A, and T2647A), which did not affect DNA-PKcs recruitment, showed delayed kinetics of disassembly from DNA lesions as compared to the wild type (Uematsu et al., 2007). Taken together, these results suggest that phosphorylation of DNA-PKcs not only affects its DNA repair activity but it also modulates DNA-PKcs dynamics on DNA breaks until repair is complete.

DNA-PKcs belongs to the PIKK family and serves as a serine / threonine protein kinase having a high preference for phosphorylating targets on SQ/TQ motifs. Even though it has a variety of downstream targets involved in many aspects of cellular mechanisms from innate immunity towards gene expression, regulation of NHEJ repair is the most essential function for DNA-PKcs. Instead of regulating itself autonomously, DNA-PKcs regulation is also mediated by ATM and ATR during genomic insults (Vidal-Eychenie et al., 2013). Upon ionizing radiation, DNA-PKcs is rapidly autophosphorylated at serine 2056 and phosphorylated at threonine 2609 by ATM (Chen et al., 2007). However, ATR also contributes towards DNA-PKcs's activity through T2609 and T2647 phosphorylation in an UV irradiation / replication stress dependent manner (Yajima et al., 2006). In addition to the interplay between PIKKs, DNA-PKcs's activity is associated with cell cycle progression upon normal conditions. It peaks during G<sub>1</sub>/S phases of the cell cycle (Lee et al., 1997), corresponding to the fact that the NHEJ repair is dominant through out the whole cell cycle, except for the

period between S and mitotic phase during which homologous recombinational repair is the preferred mechanism used.

Several lines of evidence indicate DNA *per se* is not the only determinant for DNA-PK loading to DSBs. Chromatin-associated proteins mobilize DNA-PK to chromatin in the presence of DNA damage or upon hypoxia (Bouquet et al., 2011). SIRT6, a H3K9 deacetylase, is recruited to DSB sites and forms a complex with DNA-PK (McCord et al., 2009). By contrast, cells lacking SIRT6 decrease DNA-PK occupancy on chromatin and display a defect in NHEJ repair during DSB induction (McCord et al., 2009). Other colleagues observed that oxygen-deprived hypoxia condition (0.1-1 % O<sub>2</sub>) is sufficient to induce DNA-PKcs phosphorylation on S2056 regardless of DNA lesions given that this activation doesn't cause the recruitment of DNA ligase XRCC4 on chromatin (McCord et al., 2009)(McCord et al., 2009). They found that elevated histone acetylation mediated by hypoxia is the cause of DNA-PK activation to regulate transcriptional expression of HIF-1 and GLUT1. Together, these above findings provide a different perspective that epigenetic changes, especially histone acetylation also contribute to DNA-PK's activity.

### **1.3. Cell Cycle Checkpoint**

To inherit genetic material to daughter cells accurately, mother cells duplicate the whole genome followed by cell division strategically in highly monitored and sequential phases, called the cell cycle. The cell cycle is divided into G<sub>1</sub>, S, G<sub>2</sub> and mitotic (M) phases. However, the cell cycle progression is prohibited from

entering the next phase until the previous one is completed. Such tightly-executed events are mainly coordinated through the post-transcriptional regulation of cyclin-dependent kinases known as CDKs. CDK is activated by its association with cyclin and the phosphorylation on a threonine residue in its T-loop region; conversely, the phosphorylation on threonine 14 (T14) and tyrosine 15 (Y15) residues in CDK's ATP-binding loop by Cdc25 family attenuates CDK activities (Donzelli and Draetta, 2003). CDK-cyclin complexes are also controlled by CDK inhibitor families under certain conditions (Day et al.). For example, the INK4 family, which is one of the two CKI families, interacts with CDK4 and CDK6 to promote G1-S transition whereas the other CKI family, Cip/Kip, causes cell cycle arrest at G1 phase by binding to cyclins D1-D3, CDK4, CDK6, and cyclin E (Besson et al., 2008). In addition, the timing of cell cycle checkpoints is set at G1, G2 and metaphase to guard proper cell cycle progression while cells encounter environmental stresses, replication fork stalls or DNA damage as detected by cell cycle surveillance systems. Two checkpoint kinases, Chk1 and Chk2, are identified in humans as core regulators of checkpoint initiation.

### **1.3.1. Checkpoint Kinase 1 (Chk1) in the DNA Damage Response**

Human Chk1, which is a functional counterpart of Rad53 in yeast, serves as an essential serine / threonine protein kinase for maintaining genome integrity and cell cycle arrest. Deletion of Chk1 gene in mice causes embryonic lethality while Chk1 inhibition in cells leads to increased origin firings of replication and accumulation of ssDNA, suggesting a critical role of Chk1 in S phase progression (Petermann et al., 2010). Chk1 not only controls intra-S phase progression but

also arrests G<sub>2</sub>/M phase transition upon DNA damage through negative regulation of the Cdc25 dephosphatase to elevate Wee1-mediated Cdk1 inhibitory phosphorylation on threonine 14 and tyrosine 15 residues (Cdk1<sub>T14p/Y15p</sub>) to prevent cells from premature mitosis entry (Petermann et al., 2010; Xiao et al., 2003).

Chk1 activation is usually confined to the presence of ssDNA which is rapidly coated by RPA to form a large complex with ATRIP-ATR, 9-1-1 complex, TopBP1 and Claspin. The interaction between Claspin and Chk1 on chromatin allows ATR to phosphorylate Chk1 on its C-terminal serine residues including serine 317 (Chk1<sub>S317p</sub>) and serine 345 (Chk1<sub>S345p</sub>) (Liu et al., 2006). Following DNA damage, partially phosphorylated Chk1 dissociates from chromatin and translocates to the cytoplasm where it interacts with the 14-3-3 protein to facilitate G<sub>2</sub>/M cell cycle arrest in a phosphorylation-dependent manner (Jiang et al., 2003; Smits et al., 2006). Intriguingly, immobilized-Chk1 fused with H2B failed to halt DNA damage-treated Chk1-deficient cells at G<sub>2</sub>/M phase (Smits et al., 2006), emphasizing the importance of Chk1 in communicating between chromatin and regions outside of the nucleus.

Several lines of evidence suggest Chk1 is involved in the homologous recombination (HR) process, which is further discussed in chapter 1.2. It has been shown that depletion of Chk1 sensitized cells to hydroxyurea (HU) and camptothecin (CPT) treatment and reduced the frequency of HR-directed DNA repair (Sørensen et al., 2005). Chk1 interacts with, and directly phosphorylates, Rad51 on threonine 309 (Rad51<sub>T309p</sub>) to promote Rad51 nucleofilament

formation. Either Chk1 inhibition or expression of non-phosphorylatable Rad51<sub>T309A</sub> following hydroxyurea (HU) or gemcitabine treatment gives rise to a reduction in Rad51 foci, as an indicator of impaired HR (Montano et al., 2013; Sørensen et al., 2005). In addition, Chk1 also indirectly controls Rad51 loading onto 3' protruding ssDNA by phosphorylating the localizer of Rad51, BRCA2, on its C-terminus (Bahassi et al., 2008), but how Rad51 is being placed on ssDNA accompanied with the displacement of RPA from ssDNA requires further investigation.

### **1.3.2. Checkpoint Kinase 2 (Chk2) in the DNA Damage Response**

Unlike Chk1, Chk2 is not an essential gene in mammals but is essential in yeast (Abraham, 2004; Takai et al., 2000). Chk2 is mainly activated via ATM phosphorylation on threonine 68 (Chk2<sub>T68p</sub>) for dimerization initially and dissociates to a fully active monomer spreading throughout the nucleus (Xu et al., 2002). Other than ATM, DNA-PKcs, Polo-like kinase-3 (PLK3) as well as the PML protein have been reported to be involved in Chk2 phosphorylation in special cases (Li and Stern, 2005; Zannini et al., 2014). In terms of cell cycle regulation, Chk1 and Chk2 are partially functionally redundant given that they share some substrates: for example, p53, and CDK25A/C. In response to DSBs, human Chk1 functions strictly in S and G<sub>2</sub> phase to achieve replication fork arrest whereas human Chk2 causes G<sub>1</sub>/S and G<sub>2</sub>/M cell cycle phase arrest through degradation/translocation of CDC25A/C to prevent the dephosphorylation of Cdk2 and Cdk1 respectively.

As a DDR signal transducer, Chk2 participates in homologous recombinational repair through phosphorylating key factors, such as Brca1 and Brca2 (Day et al., 2015; Zhang et al., 2004a). Chk2-induced Brca1 phosphorylation on serine 988 is critical for Brca1 departing from Chk2 to DNA lesions, which in turn promotes homologous recombinational repair (Abraham, 2004; Lee et al., 2000) or precise end joining (Wang et al., 2006). In addition, phosphorylation of Brca2 on exon 27 through Chk2 and/or Chk1 dissociates its interaction with Rad51 (Bahassi et al., 2008), leading to the loading of Rad51 to promote strand invasion during HR repair. By working in concert with ATM, Chk2 modulates heterochromatin accessibility through phosphorylating KAP1 which subsequently allows the chromatin remodeler CHD3 and heterochromatin protein 1 $\beta$  (HP1 $\beta$ ) to dissociate from heterochromatin, relaxing local chromatin architecture to facilitate DNA repair (Bolderson et al., 2012; Dinant and Luijsterburg, 2009; Goodarzi et al., 2008). It is unclear how Chk2 is inactivated after the completion of DNA repair. Chk2 can be degraded by the proteasome or dephosphorylated via phosphatases PP2A or WIP1 or PP1 in unperturbed condition (Zannini et al., 2014). However, it needs to be elucidated whether the above components contribute to turning off DNA damage-induced Chk2 activation.

#### **1.4. Major DNA Repair Pathways in Humans**

DNA repair is a highly conserved pathway throughout evolution and has been specialized into several sophisticated repair mechanisms to cope with a variety of endogenous or exogenous genomic insults. The DSB is regarded as the most

severe damage among all kinds of DNA lesion if unrepaired, it will lead to cell death. Two main DSB repair pathways in humans are non-homologous recombination and homologous recombinational repair, which are tightly correlated with cell cycle progression. How cells determine the pathway choice is still not entirely clear.

#### **1.4.1. Non-Homologous End Joining (NHEJ)**

NHEJ is the predominant DSB repair pathway used in mammalian cells to achieve prompt, high efficient DNA repair. Ku70, Ku86 and DNA-PKcs proteins are abundant and flexible enough that they recognize various DNA end structures throughout the cell cycle that makes NHEJ advantageous for the timely prevention of genomic instability. Since DNA-PKcs was discussed in 1.2.3, here the mechanism of NHEJ will be described. During DSB induction, Ku heterodimers rapidly bind to DNA ends and tether two adjacent ends together followed by recruiting DNA-PKcs to cover the exposed end structures. Given that DNA-PKcs protects DNA from further end processing, it helps initiate NHEJ, rather than HR. Instead of DNA-PK, this pathway requires other core components including the endo/exonuclease Artemis, XRCC4-like factor (XLF)-XRCC4 and DNA ligase IV.

After DNA-PKcs autophosphorylation at the ABCDE cluster, the conformational change of DNA-PKcs allows Artemis access to DNA ends. Artemis has endo/exonuclease activities to process various types of damaged DNA overhangs and is specific to open DNA hairpins found at the DNA ends

during V(D)J recombination. However, Artemis is not the only enzyme possessing nucleolytic activities in NHEJ repair. Polynucleotide kinase/phosphatase (PNKP), AP endonuclease 1 (APE1), CtIP and tyrosyl-DNA phosphodiesterase 1 (TDP1) play roles in NHEJ as well (Lees-Miller and Meek, 2003; Povirk, 2012). As a substrate of DNA-PKcs, ATM and ATR, DNA-PKcs-mediated Artemis phosphorylation is important for its endonuclease activity. It also has been reported that Artemis phosphorylation induced by ATM is involved in G<sub>2</sub>/M cell cycle arrest after IR irradiation while ATR-mediated Artemis phosphorylation facilitates recovery from S phase arrest upon UV exposure, but the mechanism of the impact of ATM and ATR on Artemis's functions remains unclear (Chen et al., 2005; Lee et al., 2000; Zhang et al., 2004b). Once DNA end processing is complete, XLF-XRCC4 and DNA ligase IV form an essential complex to carry out the ligation step followed by disassociation of the NHEJ repair machinery.

Given the importance of NHEJ in maintaining genomic stability, evidence in knockout mice has provided clear links between NHEJ and diseases. For example, Ku deficient mice have early aging accompanied with telomere shortening (Li et al., 2007). DNA-PKcs knockout mice show severe combined immunodeficiency (SCID), resulting from failed V (D) J recombination (Taccioli et al., 1998). In addition, sporadic cancers also commonly have abnormal expression levels of Ku and DNA-PKcs (Bouchaert et al., 2012; Soderlund Leifler et al., 2010). Taken together, to date numerous studies from the perspective of DNA repair *per se* and clinical data have indicated the pivotal roles of NHEJ in



guarding genomic integrity. However, little is known about whether chromatin and/or post-translational modifications (PTMs) on histones have an impact on end joining repair. Therefore, this thesis will mainly focus on DNA repair within the chromatin context.

#### **1.4.2. Homologous Recombinational (HR) Repair**

HR repair, a repair pathway with high fidelity for various DNA lesions including radiation, genotoxic chemical treatment and replication fork collapse, is strictly regulated during S/G<sub>2</sub> phase during which homologous strands are present as a matchable template for repairing broken DNA. 3' to 5' end resection from DNA break sites generates a single-stranded region which serves as a substrate for strand invasion *in trans*. Multiple factors are involved in the process of end resection including the MRN complex, C-terminal-binding protein interacting protein (CtIP), BRCA1, EXO1 and BLM/Dna2 in eukaryotes. MRE11 possesses 5' to 3' exonuclease and endonuclease activities. It is believed that MRE11 nicks DNA by its endonuclease activity to allow the access for DNA digestion from 5' to 3' end and exposes 3' overhangs of the complementary strand (Paull and Gellert, 1998). However, it is not sufficient to carry out resection by the MRN complex alone; instead, MRE11 coordinates with CtIP and Brca1 by phosphorylating CtIP and stabilizing its levels through CDK2 and their physical associations to form the MRN-CtIP-BRCA1 complex during S/G<sub>2</sub> phase (Buis et al., 2012). The main role of CtIP in the complex is to enhance MRE11 functions as well as to recruit other repair factors and its own nuclease activity is widely

used in response to complex DNA lesions produced by irradiation, topoisomerase-mediated breaks and other nucleotide-protein adducts but is dispensable for repair of restriction enzyme-induced clean DNA ends (Makharashvili and Paull, 2015; Makharashvili et al., 2014; Wang et al., 2014). Furthermore, a two-step model has been proposed that following the initial end resection, EXO1 and BLM are required for further extensive resection to generate a longer 3' tail (Nimonkar et al., 2011) which is needed for efficient RPA binding (Fanning et al., 2006). Cells lacking either EXO1 or BLM have minimal effects on CPT sensitivity and RPA focus formation, which become more severe upon double-depletion, suggesting a redundant but critical role of EXO and BLM in the process of end resection (Karanja et al., 2012).

RPA has a very high affinity for ssDNA (dissociation constant  $K_d$  is between  $10^{-10}$ ~ $10^{-8}$  M) which is greatly dependent on the length of DNA (Iftode et al., 1999) RPA-coated ssDNA not only protects its integrity but serves as a platform to recruit various factors, for example, BRCA1, BRCA2 and PALB2 during the process of HR repair. Paradoxically, RPA binding is highly dynamic so as to easily remove RPA from ssDNA, possibly through concentration-dependent exchange with other ssDNA-binding proteins such as Rad51 which has been proven *in vitro* (Gibb et al., 2014), but little is known about the exchange mechanism *in vivo*.

To initiate strand invasion, Rad51 has to form nucleofilament on ssDNA following the displacement of RPA. Given that Rad51 shows a higher specificity to duplex DNA tailed with ssDNA than ssDNA alone (Mazin et al., 2000), the

localization of Rad51 to resected 3' DNA tails requires assistance from other components. Studies in yeast provided strong evidence that Rad52 targets Rad51 to ssDNA in a competitive situation with RPA (New et al., 1998; Shinohara and Ogawa, 1998), RAD52 null mice displayed minimal effects on HR phenotypes, DNA damage sensitivity, viability and fertility (Rijkers et al., 1998), but RAD52 inactivation became synergistically lethal with BRCA1 and PALB2 as well as BRCA2 deficiencies and showed severe chromosomal fragility as well as dramatic reduction in Rad51 focus formation (Feng et al., 2011; Lok et al., 2013). BRCA2 is also one of the important HR proteins that can load Rad51 onto ssDNA through its BRC repeats and C-terminal Rad51 binding domain to displace RPA. BRCA2 requires PALB2 that contains a chromatin-association motif (Bleuyard et al., 2012) to serve as a scaffold to form the BRCA complex on chromatin (Sy et al., 2009). Even though a few theories have been proposed, how RPA is displaced from ssDNA is still poorly understood.

More and more histone modifiers and remodelers including yeast RSC (human SMARCAD orthologues), nucleolin (Goldstein et al., 2013), INO80 and SWI/SNF1 have been reported to participate in navigation of the chromatin structure during the process of DNA break end repair. These above remodelers slide or remove nucleosomes away from DNA lesions to allow the access of repair factors. For instance, cells devoid of mammalian INO80 failed in the timely removal of H2A.Z from damaged chromatin, leading to reduced generation of ssDNA, leading to impaired HR (Alatwi and Downs, 2015; Gospodinov et al., 2011). Furthermore, long-range DNA resection requires yeast Fun30 or human

SMARCAD1 to either take off histones or de-compact nucleosomes, because otherwise HR would not be able to proceed (Costelloe et al., 2012).

### **1.5. Chromatin Dynamics in DNA Damage Response**

Chromatin is composed of numerous nucleosomes that can be regarded as an obstacle towards genomic processes in certain situations. A growing number of studies demonstrate the increase of DNA accessibility within the chromatin, facilitated by chromatin remodelers, histone modifiers as well as histone chaperones, promotes DNA repair (Price and D'Andrea, 2013). In addition, the processes of chromatin disassembly and assembly during DSB repair are important for cell viability in yeast (Linger and Tyler, 2007; Tyler et al., 1999). The first discovery of nucleosome disassembly was observed during nucleotide excision repair of UV-induced DNA damage (Smerdon and Lieberman, 1978). The removal of histones from around a DSB provides evidence of chromatin disassembly to increase the accessibility for the DNA repair machinery (Tsukuda et al., 2005). However, the influence of chromatin disassembly from a DSB is a reciprocal reaction in which NBS1 and ATM are required for histone removal (Berkovich et al., 2007). Following the completion of DNA repair, chromatin structure around the DSB was restored by ASF1/CAF-1-mediated chromatin reassembly (Chen et al., 2008; Li and Tyler, 2016). The absence of chromatin assembly after DNA repair led to the persistent activation of the DNA damage checkpoint kinase, Rad53, in yeast, suggesting an important but still elusive function of histone loading to turn off DDR signals (Chen et al., 2008). To

date, the current knowledge for DNA repair in the context of chromatin is the “access-repair-restore” model (Polo and Almouzni, 2015; Soria et al., 2012) (**Fig. 2**). The DSB repair machinery must be able 1. to recognize DNA lesions within various chromatin structure to initiate the DDR. 2. to unfold chromatin by rearranging or evicting local histones around breaks, which allows the access of repair factors to damage sites. After DNA repair is complete, chromatin architecture will be restored to ensure the chromosomal integrity and epigenetic complexity. The fundamental concept of chromatin dynamics relies on the equilibrium of nucleosome disassembly and assembly. However, given the fact that chromatin disassembly and assembly are highly intertwined with repair processes and DDR signaling, it requires further investigations as to whether the chromatin spatial rearrangement is important in humans during DSB repair and when chromatin reassembly occurs after DNA damage.

Figure 2

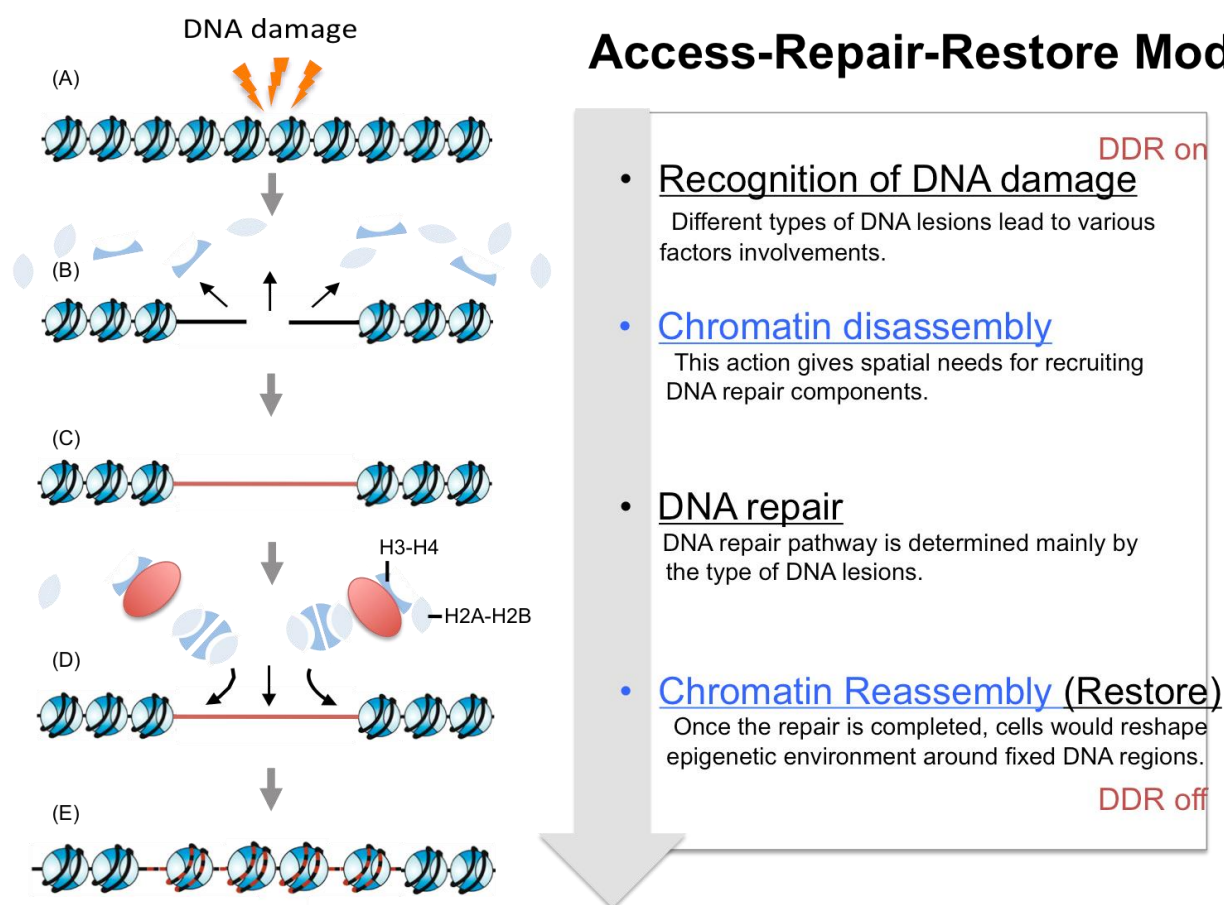


Figure 2. The Access-Repair-Restore model. DNA damage and repair occur in the context of chromatin. To overcome the chromatin barrier, cells have to (A) first recognize types of DNA lesions for initiating proper cellular responses and allowing (B) chromatin modifiers/remodelers to rearrange local chromatin architecture by which (C) repair machineries will easily get

**access to break sites. (D)(E) Histone chaperones will restore the chromatin during/after completion of repair.**

#### **1.5.1. Nucleosome remodelers**

There are four families of ATP-dependent nucleosome remodeler complexes including SWI/SNF, ISWI, CHD and INO80 (Becker and Workman, 2013). All of these families contain a DNA-dependent ATPase domain to hydrolyze ATP as a source of energy to break histone-DNA contacts. These remodelers play a critical role in altering chromatin structure by moving or sliding histones, changing compositions of histone octamer or facilitating nucleosome spatial arrangements to cope with various genomic processes. How those remodelers contribute to chromatin structure depends on their own specialization. For example, SWI/SNF and CHD are responsible for sliding and ejecting histone octamers on gene promoters to regulate transcriptional activities (Marfella and Imbalzano, 2007). The ISWI family functions in spacing nucleosome arrays which gives rise to transcriptional repression (Badenhorst et al., 2002). Chromatin remodelers also participate in DNA repair. SWI/SNF, INO80 and SMARCD1 (See **Section 1.4.2.**) were shown to have clear roles in homologous recombinational repair and CHD1 deficiency causes impeded DNA repair (Kari et al., 2016).

#### **1.5.2. Histone chaperones**

Histone chaperones are a specialized group of proteins that generally don't have enzymatic activities but have two important common characteristics that are to bind histones and to regulate nucleosome assembly / disassembly (Burgess

and Zhang, 2013). Histone chaperones can be categorized as H2A-H2B and H3-H4 chaperones depending on which core histones they are associated with. However, some of them can interact with both H2A-H2B and H3-H4, for example, FACT protein. Histone chaperones have several characteristics, but may not be applicable to every chaperone. First, histone chaperones shuttle histones from the cytoplasm to nucleus, for example, ASF1 and NASP proteins. Second, histone chaperones serve as a reservoir to either store extra histones or to buffer excess histones to secure enough histone supply, which is a property of ASF1 and NASP. Third, histone chaperones such as ASF1 can synergize with other downstream chaperones or collaborate with some histone modifiers to deliver histones or regulate post-translational modifications (PTMs) on histones, respectively. Lastly, histone chaperones mediate chromatin disassembly and reassembly (Burgess and Zhang, 2013). Indeed, histone chaperones participate in genomic processes including DNA replication, repair and transcription. This thesis will cover three chaperones, as follows.

#### **1.5.2.1. Anti-Silencing Function 1 (ASF1)**

Asf1 is named after identifying one of its functional characteristics that is to suppress the silence of mating type loci in budding yeast when overexpressed (Roos et al., 1997). However, despite the initial finding in a potential role in transcriptional activation, ASF1 was identified biochemically as a H3-H4 chaperone that functions as a chromatin assembly factor (Tyler et al., 1999). Unlike yeast and *Drosophila*, which have only one Asf1 gene, two paralogs of



ASF1, ASF1A and ASF1B, which share a highly conserved N-terminal domain but are very diverse in their C-terminal acidic tails, were found in mammals.

In terms of DNA replication, origin firing and replication fork movement requires numerous proteins to open up the chromatin and unwind the DNA, allowing the DNA replication machinery to proceed. Studies demonstrate naked stretches of DNA, which are considered to lack nucleosomes, extend about 300 bp behind and ahead of the replication fork (Gruss et al., 1993). How cells regulate nucleosome disruption in this case remains elusive. It has been discovered that ASF1 hands newly-synthesized H3.1-H4 over to another CAF-1 which interacts with PCNA during DNA replication (**Fig. 3**) to execute histone deposition on newly-synthesized DNA (Park and Luger, 2008). Newly-synthesized histones are usually acetylated before their assembly including acetylation of H3 lysine 56 (H3K56ac) (Das et al., 2009). This process requires mammalian ASF1 presenting new H3 to the pan-acetyltransferase CBP/p300 and then acetylated H3 is passed through CAF-1 onto replicated DNA. Moreover, ASF1 forms a complex with MCM2-7 at replication sites where it is thought to be responsible for transferring recycled parental histone H3.1-H4 behind the replication fork to the newly synthesized DNA (Groth et al., 2007).

As an upstream histone chaperone, ASF1 donates H3.3, which is a replication-independent H3 variant, to HIRA for transcription-coupled chromatin assembly (**Fig. 3**) (Park and Luger, 2008). The enrichment of H3.3 on the genome corresponds to the profile of RNA polymerase II (RNA PolII) distribution along the genome, implying that H3.3 incorporation occurs during transcriptional

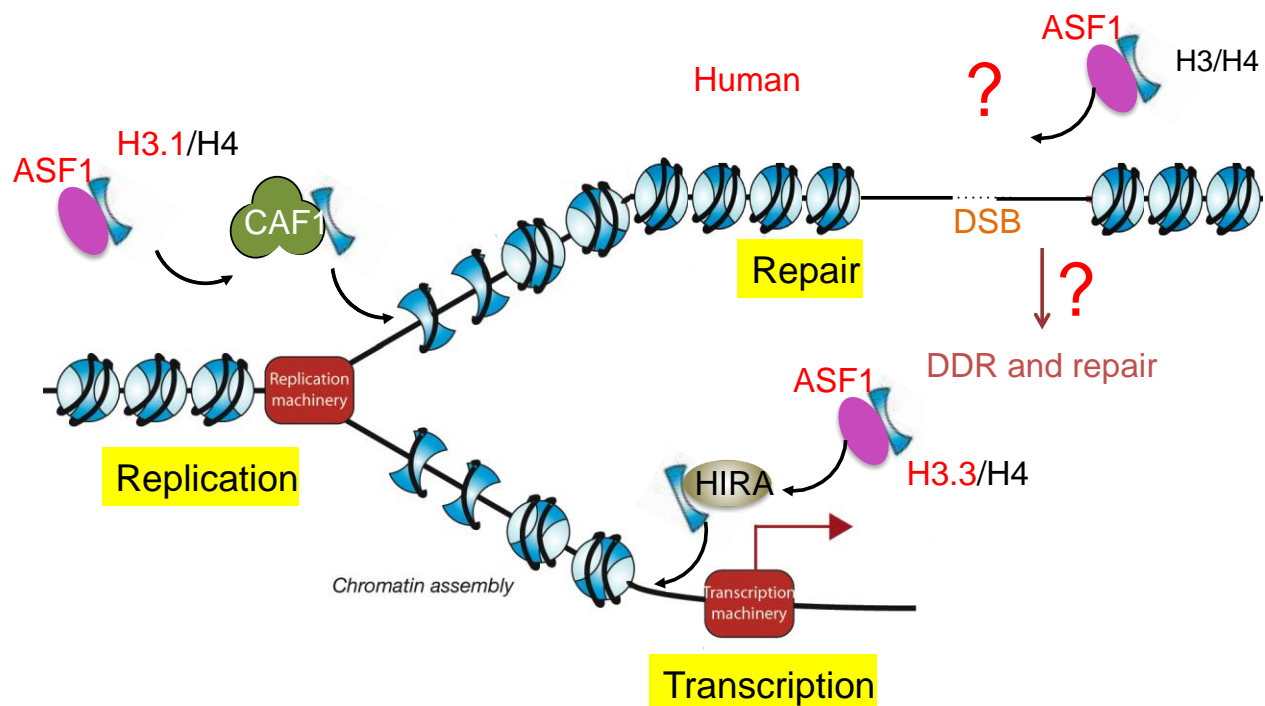
elongation and is required for transcriptional regulation (Schwartz and Ahmad, 2005); mechanistically, in budding yeast, Asf1 associates with promoter / coding regions and assists RNA PolII loading onto chromatin. Interestingly, Asf1 is responsible for both H3 eviction and incorporation during elongation (Schwabish and Struhl, 2006). Meanwhile, using Chromatin immunoprecipitation (ChIP) against H3K56ac, it shows Asf1-mediated acetylation of H3 on lysine 56 is enriched at promoter regions and facilitates chromatin destabilization at the *PHO5* promoter region in yeast. In agreement, both *ASF1* deletion and non-acetylatable H3K56R mutant strains displayed higher levels of H3 at the *PHO5* promoter and transcriptional regression (Williams et al., 2008), suggesting that acetylation of H3K56 promotes local chromatin accessibility via Asf1-mediated deposition of newly synthesized H3.

In terms of maintaining genomic integrity, our lab has demonstrated in yeast that *ASF1* deletion mutants are highly sensitive to DNA damaging agents (Tyler et al., 1999). We previously showed that Asf1 participates in the recovery of the yeast DNA damage checkpoint kinase Rad53 which is a functional ortholog of human Chk1 (Kumar and Burgers, 2013) through mediating nucleosome deposition after double-strand break (DSB) repair (Chen et al., 2008), but little is known about whether and how ASF1 impacts human genome stability and DDR signal transduction.

Taken together, both isoforms of ASF1 in humans share common features in buffering excess histone H3.1 and function in concert with CAF-1 to facilitate replication-coupled chromatin assembly, suggesting that they may possibly

functionally substitute for each other. Little is known about the function of ASF1B alone. Recently, some reports indicate that ASF1B plays specific roles in breast cancer proliferation (Corpet et al., 2011) as well as human beta cells proliferation via depositing H3.3 onto DNA (Paul et al., 2016). ASF1B is also required for promoting reproductive capability concomitant with its expression during meiotic entry in female mice (Messiaen et al., 2016). ASF1A promotes senescence by mediating SAHA foci formation with assistance from another histone chaperone, HIRA (Yamane et al., 2011). Therefore, it is possible that the distinct signatures between two the two ASF1 isoforms result from their flexible C-terminal domains which may determine binding partners or affect histone associations in various cellular conditions.

**Figure 3**



**Figure 3. Outline of ASF1 functions in all genomic processes. ASF1 cooperates with downstream histone chaperones to participate in depositing histones onto naked DNA during replication, transcription, as well as DNA repair which has been demonstrated in yeast. Whether ASF1 or other histone chaperones will affect the DDR or DSB repair is unclear in human cells.**

#### **1.5.2.2. Chromatin assembly factor-1 (CAF-1)**

CAF-1 is composed of three subunits called p150, p60 and p48 in human cells. During replication, CAF-1 forms a complex with PCNA at replication forks to promote chromatin assembly via facilitating H3.1 incorporation onto nascent DNA (Shibahara and Stillman, 1999). Upon various genomic insults, human cells lacking CAF-1 fail to put nucleosomes onto replicated regions and are halted at S phase during cell cycle progression (Hoek and Stillman, 2003). Recent studies using the SNAP-tagging method to distinguish old and new histones supports a role for CAF-1 in depositing newly-synthesized H3.1 during nucleotide excision repair (Adam et al., 2013). Furthermore, studies from several species demonstrate CAF-1 participates in HR repair (Lewis et al., 2005; Linger and Tyler, 2005; Song et al., 2007). For example, p150 CAF-1 is recruited to DNA lesions either to stabilize resected ends or to help recruitment of Rad51 via the

interaction with HP1 $\alpha$  (Baldeyron et al., 2011). Other studies using yeast revealed the possibility that reduced nucleosome assembly activity in CAF-1 deletion mutants may give rise to reduced recombinational repair efficiency (Lewis et al., 2005; Song et al., 2007). Song, et al. found the p180 mutant, which is a counterpart of human p150 in *Drosophila* has a defect in HR repair which is rescued by complementation of human p150 (Song et al., 2007). However, whether maintenance of genome stability is directly impacted by CAF-1 *per se*, or is indirectly promoted by the CAF-1 mediated proper epigenetic environment requires further investigation.

#### **1.5.2.3. Histone regulator A (HIRA) complex**

The human HIRA complex has three subunits including HIRA, UBN1 and CABIN1 (Rai et al., 2011). HIRA protein receives H3.3/H4 from ASF1, and it mediates H3.3/H4 deposition into transcriptionally active promoters and within gene bodies, behind RNA polymerase II machinery (Pchelintsev et al., 2013). Recent studies also indicate that HIRA participates in maintaining global chromatin structure and nucleosome occupancy over the whole genome (Gal et al., 2015). In light of this function, H3.3 incorporation mediated by the HIRA complex is responsible for transcription restart after completion of nucleotide excision repair (Adam et al., 2013) and is also important for mouse development (Lin et al., 2014). During NHEJ, we recently found that HIRA also promotes chromatin assembly onto the repaired DNA in human cells (Li and Tyler, 2016).

### 1.5.3. Functional characteristics of the MMS22L/TONSL complex

Mms22 was first identified as a repair protein from a yeast mutant screen with hypersensitivity to methylmethanesulphonate (MMS) (Prakash and Prakash, 1977). Deletion of yeast *MMS22* causes replication fork stalling, spontaneous DNA damage and impeded HR (Vaisica et al., 2011). Given that yeast Mms22 interacts genetically in the same pathway with the histone chaperone Asf1, the acetyltransferase Rtt109, the cullin protein Rtt101, Rtt107, replisome and Mms1 (Collins et al., 2007), it is believed that Mms22 may promote DNA replication in a manner potentially related to chromatin. The Daniel Durocher and John Rouse laboratories found Mms22-like (MMS22L) protein, the Mms22 orthologue in humans, based on an siRNA screen and blast for homologue analysis, respectively, and that it associates with both ASF1 isoforms, RPA complex, MCM complex, histones and the histone chaperone FACT (Duro et al., 2010; O'Donnell et al., 2010). Consistent with observations in yeast, MMS22L shares common features with yeast Mms22 in maintaining genomic integrity because cells lacking MMS22L show G<sub>2</sub>/M phase arrest, long-lasting 53BP1 foci and impaired HR pathway caused by defective RAD51 nucleofilament formation (Duro et al., 2010; O'Donnell et al., 2010). Different from yeast Mms22, MMS22L tightly interacts with the TONSL protein that is an orthologue of plant TONKTSU. Intriguingly, ASF1 and H3/H4 remain part of the TONSL/MMS22L complex, implicating that histone assembly during HR repair may be conserved from yeast to humans. Their findings conclude that MMS22L/TONSL may localize at ssDNA to facilitate Rad51 loading (Abraham, 2004; Duro et al., 2010) (O'Donnell et al., 2010). In

agreement, a recent study further supports this conclusion by *in vitro* analysis, as MMS22L/TONSL associates with RPA-coated ssDNA and directly interacts with Rad51 via MMS22L (Piwko et al., 2016). Moreover, TONSL also recognizes unmethylated H4 at K20 which is considered as a marker of newly-synthesized H4 at the replication fork and DNA lesions through its ankyrin repeat domain (Bleuyard et al.) (Saredi et al., 2016). As a result, we propose that newly-deposited nucleosomes around DNA break ends may facilitate MMS22L/TONSL complex recruitment, but how MMS22L/TONSL interplays with RPA and histones on DNA lesions requires further investigation.

## **Chapter 2**

### **Materials and methods**



### 2.1. Cell culture, antibodies, DNA damaging agent treatments

HeLa and human erythroleukemia K562 cells were grown in complete DMEM and RPMI (Roswell Park Memorial Institute) 1640 Medium at 37°C with 5% CO<sub>2</sub>, respectively. For transient transfection, 2 x 10<sup>6</sup> cells were seeded per 60mm dish for 24 hours prior to the delivery of plasmid DNA using Lipofectamine 2000 (Invitrogen). To induce DNA damage, cells were generally treated with 40 µg/ml Bleomycin (#9041-93-4, Cayman Chemical Company) and 0.5 µg/ml Neocarzinostatin (#N9162, Sigma) for 2 hours followed by harvesting at indicated time points after washing with PBS. The antibodies used in this study are listed in Table 1.

**Table1. A list of antibodies used in this study.**

<b>Antibody Name</b>	<b>Catalogue number/ company</b>	<b>Applications</b>	<b>Dilution rates</b>
ASF1A	#2990/ Cell Signaling	Immunoblotting	1:1000
ASF1B	MA5-14836/ ThermoFisher	Immunoblotting	1:750
ATM	#2873/ Cell Signaling	Immunoblotting	1:1000
ATR (H-300)	sc-1887/Santa Cruz	Immunoblotting/ immunofluorescence	1:1000/ 1:600
BRCA2	GTX70121/ GeneTex	Immunofluorescence	1:400
CAF-1 p150	sc-133105/Santa Cruz	Immunoblotting	1:1000
CAF-1 p60	A301-085A/	Immunoblotting	1:1000

	BETHYL		
Cdc2	GTX108120/ Genetex	Immunoblotting	1:3000
Chk1	sc-8408/Santa Cruz	Immunoblotting	1:1000
DNA-PKcs	#4602/ Cell Signaling	Immunoblotting	1:1000
GAPDH	ab8245/ Abcam	Immunoblotting	1:10,000
HA	MMS-101R/ Covance	Immunoblotting	1:3000
HIRA	#39557/ Active Motif	Immunoblotting	1:750
Histone H3	ab1791/ Abcam	Immunoblotting	1:6000- 10,000
Histone H4	#39269/ Active Motif	Immunoblotting	1:1000
KAP1	GTX102226/ GenTex	Immunoblotting	1:3000
MMS22L	ab181047/ Abcam	Immunoblotting	1:1500
Orc2	#51-6875GR/ BD Biosciences	Immunoblotting	1:1500
Rad51	ab213/ Abcam	Immunoblotting/ immunofluorescence	1:1000/ 1:600
RPA34	NA19L/ Calbiochem	Immunoblotting/ immunofluorescence	1:3000/ 1:1000
TONSL	ab101898/ Abcam	Immunoblotting	1:3000
□□□□□	Ab97351/ Abcam	ChIP	
$\alpha$ -tubulin	T9026/ Sigma- Aldrich	Immunoblotting	1:5000
H3K56ac	#39281/ Active motif	Immunoblotting	1:2000

H4K16ac	#05-1232/ Millipore	Immunoblotting	1:1000
H4K8ac	#CS205840/ Millipore	Immunoblotting	1:1000
Phospho-ATM S1981	#200-301-400/ Rockland	Immunoblotting/ immunofluorescence	1:1000/1:800
Phospho-cdc2 (Tyr15)	#9111/ Cell Signaling	Immunoblotting	1:1000
Phospho-Chk1 S345	#2341/ Cell Signaling	Immunoblotting	1:1000
Phospho-Chk1 S317	#2360/ Cell Signaling	Immunoblotting	1:1000
Phospho-RAD9 S328	AP3225a/ Abgent	Immunoblotting	1:1000
Phospho-DNA- PKcs T2609	ab18356/ Abcam	Immunoblotting/ immunofluorescence	1:1000/ 1:800
Phospho-DNA- PKcs S2056	ab18192/ Abcam	Immunoblotting/ immunofluorescence	1:2000/ 1:800
Phospho-KAP1 S824	A300-767A BETHYL	Immunoblotting/ immunofluorescence	1:2000/ 1:800
Phospho-RPA 32 S4/S8	A300-245A BETHYL	Immunoblotting	1:3000

## 2.2. siRNA and shRNA knockdowns

Sequences for small RNAs against ASF1A and ASF1B were described in a previous study (Groth et al., 2005) and purchased from Dharmacon. 100 nM of siASF1A and siASF1B oligonucleotides were mixed with INTERFERin siRNA transfection reagent (#409-10, Polyplus transfection) according to the manufacturer's protocol to perform ASF1A/B knockdowns for 48 hours. The

siRNA control was a random scrambled RNA sequence. shRNA lentiviral plasmids against ASF1A, ASF1B, HIRA, CAF-1, DNA-PKcs, Rad51 and the control were obtained from the shRNA and ORFeome Core Facility at the University of Texas MD Anderson Cancer Center (Houston, TX). MMS22L, CtIP and 53BP1 shRNA plasmids were provided through Memorial Sloan Kettering Cancer Center (MSKCC) RNAi core (New York, NY). 293T cells were transfected with shRNA plasmids and the viral packaging vectors pCMV-VSVG and pCMV-dR8.2. The culture media containing shRNA virus particles were collected 48 to 72 hours after transfection and stored at -80 °C. To generate shRNA knockdown cells, cells were incubated with shRNA lentiviral supernatant for 24 hours. After shRNA lentiviral infection, shRNA knockdowns were selected in 1 µg/ml Puromycin for 48 hours. The knockdown efficiency of each target protein was determined by Western blotting. Each experiment was repeated at least 3 times and representative results are shown. All siRNA and shRNA target sequences used in this study were list in Table 2.

**Table 2. A list of siRNAs and shRNAs used in this study.**

<b>siRNA</b>	<b>clone ID</b>	<b>Company</b>	<b>Target nucleotide sequence</b>
scrambled	D001206-13-05	Dharmacon	n.a.
ASF1A	n.a.	Dharmacon	5' AAUC CAGGACUCAUUCCAGAU 3'
ASF1B	n.a.	Dharmacon	5' AACAAACGAGUACCUCAACCCU 3'
<b>shRNA</b>			
scrambled	RHS4346	Dharmacon	n.a.
ASF1A	V3LHS_351239	Dharmacon	5' TAGATGCCAAAATATTCCT 3'
ASF1B	V3LHS_410407	Dharmacon	5' TAGAACTGAAGTACCTGCT 3'
CAF-1 (p150)	V2LHS_248088	Dharmacon	5' TCAAGAAGGAGAACTCGCC 3'
HIRA	V2LHS_171670	Dharmacon	5' TAGAGCATACCAAGATGCC 3'
DNA-PKcs-2	V2LHS_231208	Dharmacon	5' ATTTCTTGAATCCATGACC 3'
DNA-PKcs-3	V2LHS_94774	Dharmacon	5' ATGATTCAGACTTTCACTG 3'
Rad51	V3LHS_318594	Dharmacon	5' TTGGCTTCACTAATTCCCT 3'
MMS22L	TRCN0000149282	Sigma-Aldrich	5' GCACCTATGTTGTCAGCAATT 3'
CtIP	TRCN0000005406	Sigma-Aldrich	5' CGTCAGCCTTACAACGCAATA 3'
53BP1	TRCN0000018869	Sigma-Aldrich	5' CCCTTGTTTCAGGACAGTCTTT 3'

### **2.3. Alkaline comet assay**

2 x 10<sup>5</sup> K562 cells were pelleted and washed in 1 x PBS followed by resuspension in 1ml 1 x PBS buffer. 1 x 10<sup>4</sup> cells were added to 45 °C pre-warmed low melting-point agarose (#16520050, Invitrogen) to make a final 0.5 % agarose mixture and 50 µl was applied to comet slides (#4250-200-03, Trevigen). The slides were immersed in lysis solution (#4250-010-01, Trevigen) for 1 hour at 4 °C. The slides were immersed in prechilled unwinding buffer (0.3N NaOH and 1mM EDTA, pH13.1) for another 30 minutes at 4 °C followed by electrophoresis at 1.5 V cm<sup>-1</sup> for 15min at room temperature. After electrophoresis, nuclear DNA was counterstained with CyberGold. 100 cells of each sample (n=2) from x 100

magnifications images were scored to calculate tail moment (a.u.) using ImageJ software.

#### **2.4. Immunofluorescence analysis**

Cells were plated on poly-L-lysine-coated coverslips for 24 hours. After bleomycin treatment, coverslips were washed with PBS. For DNA damage foci analysis, 0.1 % Triton-X-100 in cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl<sub>2</sub>, 10 mM PIPES (pH 6.8)) was added to permeabilize cells for 3 minutes at room temperature. Cells were washed once with TBS-T (0.05 % Tween-20) and fixed with 4 % paraformaldehyde for 10 minutes. Fixed cells were blocked with 3 % BSA for 30 minutes. Primary antibodies diluted in 3 % BSA blocking buffer were subsequently incubated with coverslips overnight at 4 °C. Cells were washed 3 times with TBS-T before incubating with fluorescence conjugated-secondary antibodies for 1 hour at room temperature in the dark. After washing in PBS, cells were mounted in prolong gold antifade mountant with DAPI and kept at 4 °C in the dark. Cells were imaged using a 3i confocal microscope system or Olympus BX53 camera and were captured using via ImageJ and photoshop software.

#### **2.5. Protein extraction and immunoblotting**

After pelleting cells and washing cells once in PBS, they were resuspended in pre-chilled RIPA buffer (50 mM Tris-HCl, pH7.4, 1 mM EDTA, 0.5 mM EGTA, 1 % Triton-X-100, 0.1 % Nadeoxycholate, 0.1 % SDS, 150 mM NaCl, protease

inhibitors (complete Mini EDTA-free, #4693159001, Roche) and phosphatase inhibitors (PhosSTOP, #4906837001, Roche)) followed by sonication. The cell debris was removed by centrifugation at 14,000 *r.p.m.*, at 4 °C for 10 minutes. Supernatants were collected and protein concentrations quantitated by Bradford protein assay (#500-0006, Bio-Rad). Equivalent protein amounts were loaded on SDS-PAGE or Criterion 4-12 % precast gels (#5671085, Bio-Rad). After electrophoresis, proteins were transferred to Nitrocellulose membrane (#10600048, GE Healthcare Life Sciences) for 90 minutes at 4 °C and membranes were incubated with 5 % non-fat milk for 1 hour at room temperature before overnight incubation with diluted primary antibodies at 4 °C. Enhanced chemiluminescence (ECL) Western blotting substrate (# RPN 3243, Amersham ECL, GE Healthcare Life Sciences) were used for detection, using a protein simple analysis machine.

## **2.6. Ectopic expression of wild type and mutant ASF1A**

HA tagged wild type (HA-WT) and mutant human ASF1A (HA-S192A) (a generous gift from Dr. Yuri M. Moshkin) were expressed from pcDNA3.1 (+) vector. Other mutations were generated on the HA-WT plasmid by site directed mutagenesis to make the V94R and S192D substitutions. All the above plasmids had been made immune to knockdown of endogenous ASF1A by nucleotide mutation of seed sequence “AGATGC” to “CGAGGC” (Table 2). For transient transfections, HeLa or ASF1A knockdown cells were transfected with ASF1A

plasmids using lipofectamine 2000 reagent (#11668500, ThermoFisher Scientific) for 24 hours.

## **2.7. Immunoprecipitation**

2 x 10<sup>6</sup> HeLa cells were seeded into 60mm dishes and incubated overnight. HA-tagged ASF1A plasmids (HA-WT, HA-V94R, HA-S192A and HA-S192D) were transfected into cells for 24 hours before harvesting. Cells were then lysed with pre-chilled modified RIPA buffer (replace 1 % Triton X-100 with 0.5 % NP-40) for 15 minutes on ice. To collect supernatant, lysates were centrifuged at 14,000 *r.p.m.* at 4 °C for 10 minutes. Cell lysates were precleared with Dynabeads Protein A (#10001D, ThermoFisher Scientific) for 1 hour followed by incubation with anti-HA-Agarose (#A2095, clone HA-7, Sigma-Aldrich) for 3 hours at 4 °C. After centrifugation, the HA-Agarose protein complexes were washed in RIPA buffer 3 times for 15 minutes and boiled with SDS loading buffer.

## **2.8. Chromatin fractionation analysis**

This analysis was performed as described previously with slight modification (Méndez and Stillman, 2000). Whole cell extract (WCE) samples were taken from the same batch of samples used for chromatin fractionation and prepared separately. Cells harvested from 6-well plates were re-suspended in buffer A (10 mM HEPES, pH7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.34 M sucrose, 10 % glycerol, 1 mM DTT, complete Mini (EDTA-free) protease and PhosSTOP phosphatase inhibitors) before adding an equal volume of buffer A with 0.2 % Triton X-100 and



was incubated on ice for 4.5 minutes. Cytoplasmic protein fractions were collected after centrifuge at 1,300 x g, for 4 minutes at 4 °C. The insoluble pellet, including chromatin, was washed with buffer A and then the supernatant was aspirated. The pellet was resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, complete Mini protease and PhosSTOP phosphatase inhibitors) for 15 minutes, followed by centrifugation at 1,700 x g, 4 minutes at 4 °C. Nuclear protein fractions were obtained from the supernatant. Wash pellet once in buffer B and apply S7 nuclease and RIPA buffer with 5 mM CaCl<sub>2</sub> to each sample for 10 minutes on ice, followed by sonication to solubilize. This is referred to as the “chromatin” sample.

## **2.9. Native BrdU resection assay**

Cells were seeded in 24-well plates containing coverslips overnight and were incubated with 30 µM BrdU (#550891, BD Biosciences) for an additional 48 hours followed by the treatment of DNA damaging agents (Bleomycin or CPT) for the time periods indicated in the figures. CtIP and 53BP1-depleted cells serve as negative and positive controls, respectively, for DNA end resection. After treatment, the same steps were carried out that were described in the immunofluorescence analysis except that the diluted anti-BrdU antibody (1:400, #555627, BD Biosciences) was applied to samples at room temperature for 2 hours. Cells were imaged using an Olympus BX53 microscope system and images were captured via Cell Sense and photoshop software. For measuring

BrdU intensity, 100 cells of each sample were quantitated by Fiji (ImageJ) software.

## **2.10. Real-time PCR and chromatin immunoprecipitation**

### *I-Ppol-based Inducible DSB system*

The pBABE-dd-I-Ppol (Addgene #49052) plasmid developed by Michael Kastan lab was introduced into HCT116 cells upon puromycin selection to obtain genomic-integrated stable bulk population. For DNA cutting and repair analysis, Shield-1 (Cheminpharma) was added to a final concentration of 1mM for 3 hours to stabilize dd-I-Ppol followed by another 30-minute incubation with 4mM 4-OHT (Sigma). After treatment, drugs were washed off in PBS to allow the process of DNA repair over time. DNA levels of I-Ppol sites were further quantified by real-time PCR. The mean value  $\pm$  S.E.M. at each time point was obtained from three independent experiments. The primer sequences used for detecting DNA levels were as follows: (SLCO5a1-F-cut: CCCAGTGCTCTGAATGTCAA; SLCO5a1-R-cut: CCATTCATGCGCGTCACTA). DNA levels were normalized to the value of real-time PCR using the GAPDH (GAPDH-F: TCAGCCAGTCCCAGCCCAAG; GAPDH-R: GAGAAAGTAGGGCCCGGCTAC) primer set.

### *Chromatin immunoprecipitation*

The procedure for chromatin immunoprecipitation (ChIP) was performed as described in the previous study (Li and Tyler, 2016). The antibodies against DNA-PK<sub>S2056p</sub> and XRCC4 were used. The enriched chromatin was applied to the real-time PCR analysis. The primer sequences (SLCO5a1F-Left: GCATGAATGG

ATGAACGAGAT; SLCO5a1R-Left: CAAGCTCAACAGGGTCTTCT) for real-time PCR flanked the 5' upstream region which was 200 b.p. distal from the cutting site. The NHEJ factor occupancy and the input at each time point were normalized to GAPDH. All data were normalized to the value of untreated sample as 1.

## **Chapter 3**

**Replication-dependent chromatin assembly affects PIKK  
activation and promotes the MMS22L/TONSL mediated transition  
from RPA- to Rad51-coated ssDNA during homologous  
recombination**

### **3.1. Introduction and rationale**

DNA damage responses occur in the context of chromatin within our cells, which is the nucleoprotein structure of nuclear DNA wrapped around the outside of octamers of core histones to form arrays of nucleosome (Kornberg, 1974). The packaging of DNA into chromatin helps regulate the accuracy and efficiency of the DNA repair process and the DDR in general (Xu and Price, 2011). For example, histone post-translational modifications help recruit proteins involved in the DDR to DSBs (Williamson et al., 2012). Furthermore, histones have been shown to be removed from around DSBs to enable DNA repair to occur in yeast and human cells (Berkovich et al., 2007; Chen et al., 2008; Goldstein et al., 2013; Li and Tyler, 2016). The requirement of chromatin disassembly around the DNA lesion for DSB repair implies that histones are a physical obstacle for DSB repair, as described in the access-repair-restore model (Polo and Almouzni, 2015). Intriguingly, reassembly of histones by the concerted action of the Asf1 and CAF-1 histone chaperones, after DSB repair, is required for turning off the DNA damage cell cycle checkpoint in yeast (Chen et al., 2008; Kim and Haber, 2009). The role of chromatin assembly in turning off the DNA damage checkpoint in yeast appears to be mediated via an interaction between non-histone bound Asf1 (having donated its histones to the newly-repaired DNA) and the Rad53 kinase (the equivalent of mammalian Chk2), to prevent further phosphorylation of Rad53 (Tsabar et al., 2016). Noteworthy, the interaction between Asf1 and Chk2 is not conserved in metazoans (Tamburini and Tyler, 2005) suggesting that this mechanism for checkpoint inactivation after DSB repair is likely to be yeast specific. As such, it is currently unknown whether chromatin assembly contributes to the repair of DSBs or whether

chromatin assembly plays a role in inactivation of the DNA damage checkpoint after DNA repair in metazoans.

There is circumstantial evidence supporting a link between chromatin assembly and DSB repair in mammalian cells. One of the two mammalian isoforms of Asf1, ASF1A, is required for chromatin assembly after NHEJ in human cells (Li and Tyler, 2016), although this chromatin assembly is not required for DNA repair via NHEJ *per se*. In addition, both human ASF1A and ASF1B exist in a complex with MMS22L and TONSL (Duro et al., 2010). MMS22L/TONSL is recruited to ssDNA coated with replication protein A (RPA) to facilitate efficient loading of the strand exchange protein Rad51 foci onto ssDNA (Duro et al., 2010; O'Donnell et al., 2010; Piwko et al., 2016), which is an intermediate event in homologous recombination necessary for strand invasion following replication stress or during DSB repair. Noteworthy, recruitment of TONSL/MMS22L to sites of DNA replication is mediated by the interaction between TONSL and newly-synthesized histones that lack H4 K20me (Saredi et al., 2016) that are deposited onto DNA by ASF1A and CAF-1.

In this thesis, given the evolutionary conservation of histone chaperones and chromatin structural alterations in response to genomic processes, I set out to firstly determine if the role of chromatin assembly in inactivation of the DNA damage checkpoint after DSB repair was conserved from yeast to humans. Secondly, I sought to dissect the impact of histone chaperone-mediated chromatin assembly on DNA repair. Indeed, I find persistent activation of the ATR-Chk1 pathway and conversely, faster inactivation of the ATM-Chk2 pathway upon knockdown of human ASF1. However, in contrast to yeast, persistent checkpoint activity was due to a defect in

homologous recombination upon depletion of human ASF1. Specifically, I find that assembly of newly-synthesized histones onto DNA by the ASF1A/CAF-1 pathway is required for recruitment of TONSL/MMS22L to facilitate Rad51 loading onto resected DNA to promote strand invasion. I also have uncovered a novel DSB-induced phosphorylation of ASF1A by the DNA-dependent protein kinase catalytic subunit and potentially other kinases. Mechanistically, phosphorylation of ASF1A in response to DSBs increased its interaction with histones and histone chaperones, and promoted recruitment of MMS22L/TONSL to damaged DNA, enabling Rad51 assembly onto ssDNA during homologous recombination. Taken together, these studies reveal an unexpected active role for chromatin assembly, presumably onto ssDNA, during a key mechanistic stage in the midst of homologous recombination.

## **3.2. Results**

### **3.2.1. Cells lacking ASF1A or CAF-1 have prolonged Chk1 phosphorylation and short lasting phosphorylated Chk2 after DSB induction, leading to a delay in cell cycle progression.**

Histones H3 and H4 are rapidly deposited onto DNA following local microirradiation-induced DNA damage in human cells (Luijsterburg et al., 2016), suggesting that chromatin is reassembled after DSB repair in humans. Indeed, ASF1A and CAF-1 mediate the local chromatin reassembly during NHEJ mediated repair of a DSB induced by the I-PpoI endonuclease in human cells (Li and Tyler, 2016). Given that chromatin assembly after DSB repair is required for inactivating the DNA damage checkpoint in yeast (Chen et al., 2008), I asked if

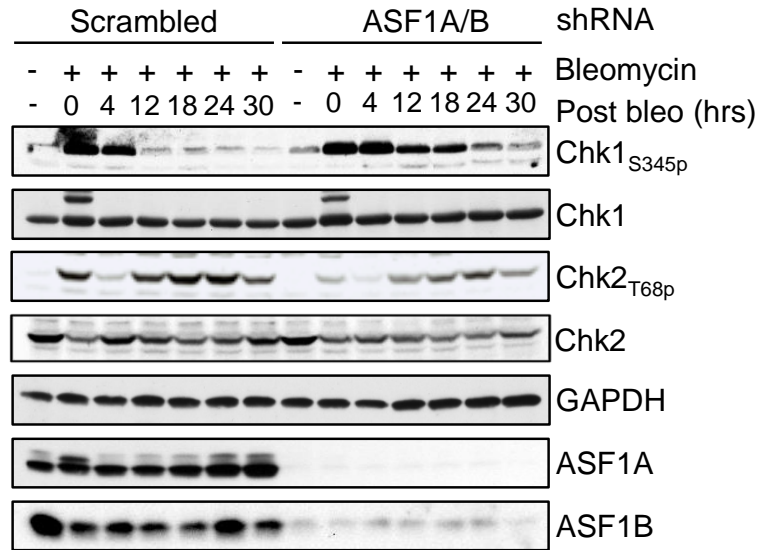
that was also the case in human cells. I induced global DSBs by treatment of HeLa cells with the radiomimetic bleomycin, followed by washing out the bleomycin (time point 0) and collecting samples at increasing time points after washing out the bleomycin (“post bleo”) (**Fig. 4A**). I knocked down expression of ASF1A and ASF1B, using a random scrambled shRNA as a control. In all experiments, the samples that were not exposed to bleomycin “-“, were incubated for the same length of time as the longest recovery time after bleomycin treatment in each experiment, to control for the potential effect of extensive periods of ASF1A/B knockdown on other cellular processes, such as DNA replication. For example, knockdown of ASF1A/B in U-2-OS cells has been shown to lead to RPA accumulation due to defective DNA replication (Groth et al., 2007) I examined DNA damage checkpoint activation and inactivation during the bleomycin treatment and recovery in the presence or absence of ASF1A/B, by western blotting for ATR-mediated phosphorylation of the Chk1 kinase, which is mainly responsible for the G<sub>2</sub>/M checkpoint (Liu et al., 2000) and for ATM-induced phosphorylation of the Chk2 kinase. In contrast to the scrambled control which rapidly inactivates Chk1, I observed persistent phosphorylation of Chk1 at Ser345 (Chk1<sub>S345p</sub>) after removal of bleomycin in ASF1 depleted cells. By contrast, Chk2 phosphorylation at Threonine 68 in the control has a slower kinetics of disappearance, and Chk2 phosphorylation weakened faster in the absence of ASF1 during the recovery from DSB damage (**Fig. 4A**). In agreement, siRNA knockdown of ASF1A/B also led to persistent Chk1 and weaker Chk2 phosphorylation in response to DNA damage (**Fig. 4B**). This



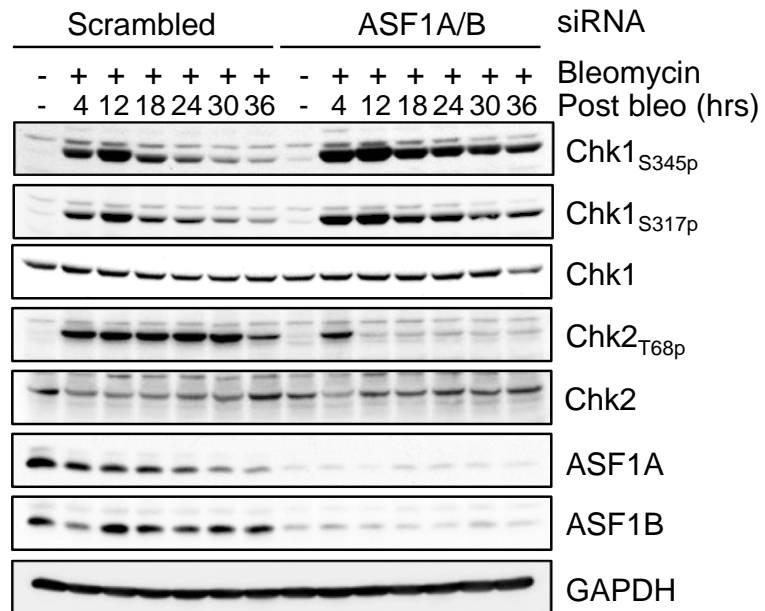
response was not unique to bleomycin, because cells challenged with another radiomimetic drug, neocarzinostatin (NCS), also showed persistent activation of Chk1 and lower levels of Chk2<sub>T68p</sub> in the absence of ASF1 (**Fig. 4C**). The dysregulation of Chk1 and Chk2 phosphorylation in the absence of ASF1A/B does not appear to reflect stabilization / degradation of either protein, because there was no difference in Chk1 and Chk2 protein half-life upon inhibition of translation with cycloheximide (**Fig. 4D**). Knockdown of ASF1A alone, but not ASF1B led to the same degree of persistent Chk1 activation; however, both ASF1 isoforms contribute to the maintenance of Chk2 activation after DNA damage (**Fig. 4E**), suggesting that the ASF1A isoform was required for a normal DNA damage response and ASF1B isoform mainly involved in ATM-Chk2 pathway. In agreement, the persistent Chk1 activation seen upon ASF1A/B knockdown was rescued by expression of exogenous ASF1A alone while Chk2 phosphorylation was still reduced (**Fig. 4F**). Taken together, these experiments indicate that ASF1A is required for timely inactivation of the ATR-Chk1 checkpoint response and both ASF1A and ASF1B participate in maintaining the activation of ATM-Chk2 pathway following induction of DSBs in human cells.

**Figure 4**

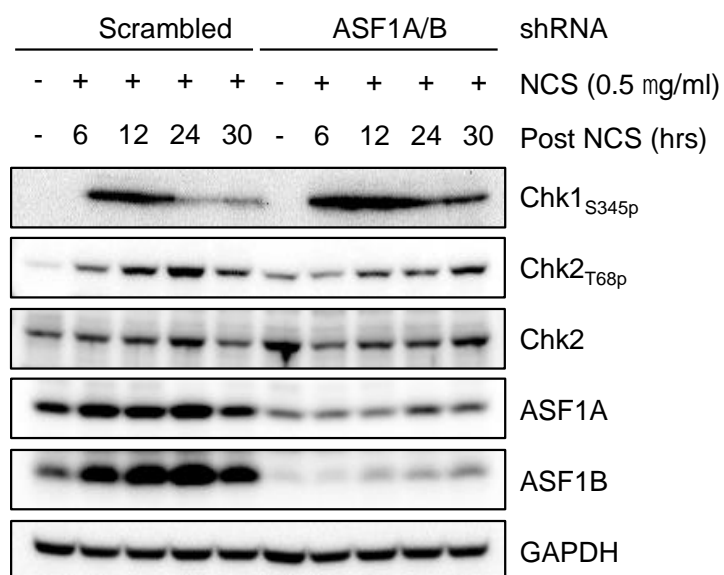
**A**



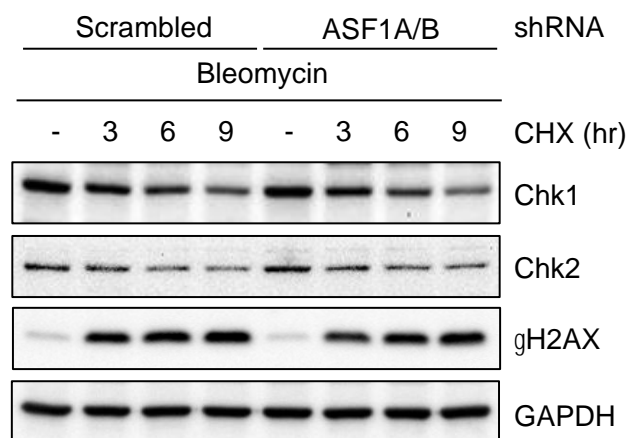
**B**



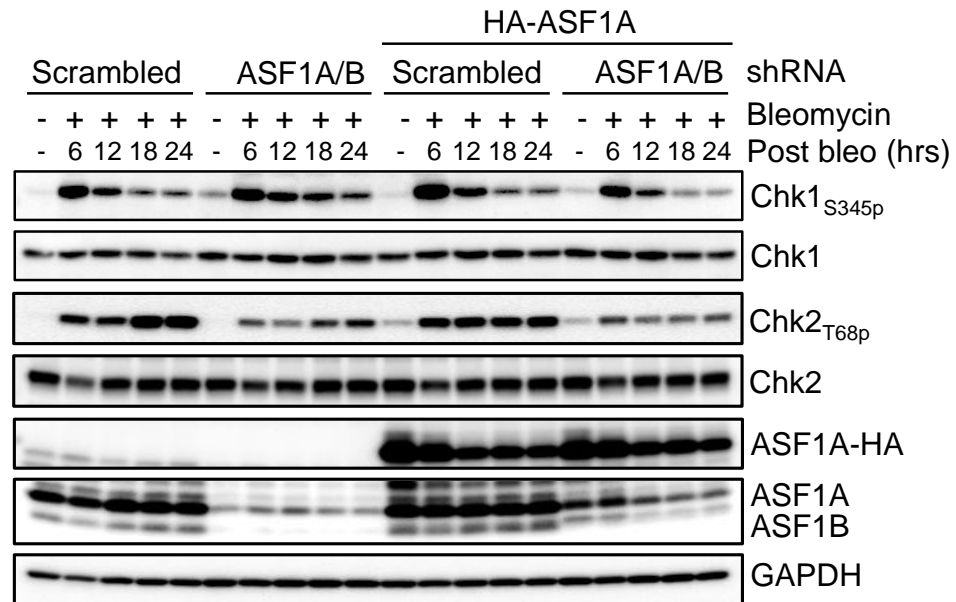
**C**



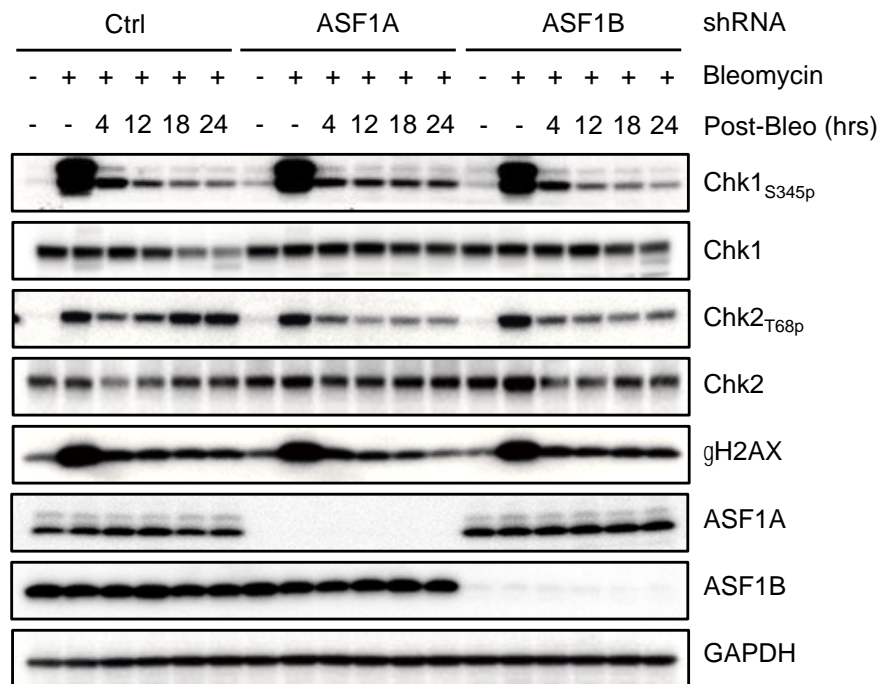
**D**



**E**



**F**



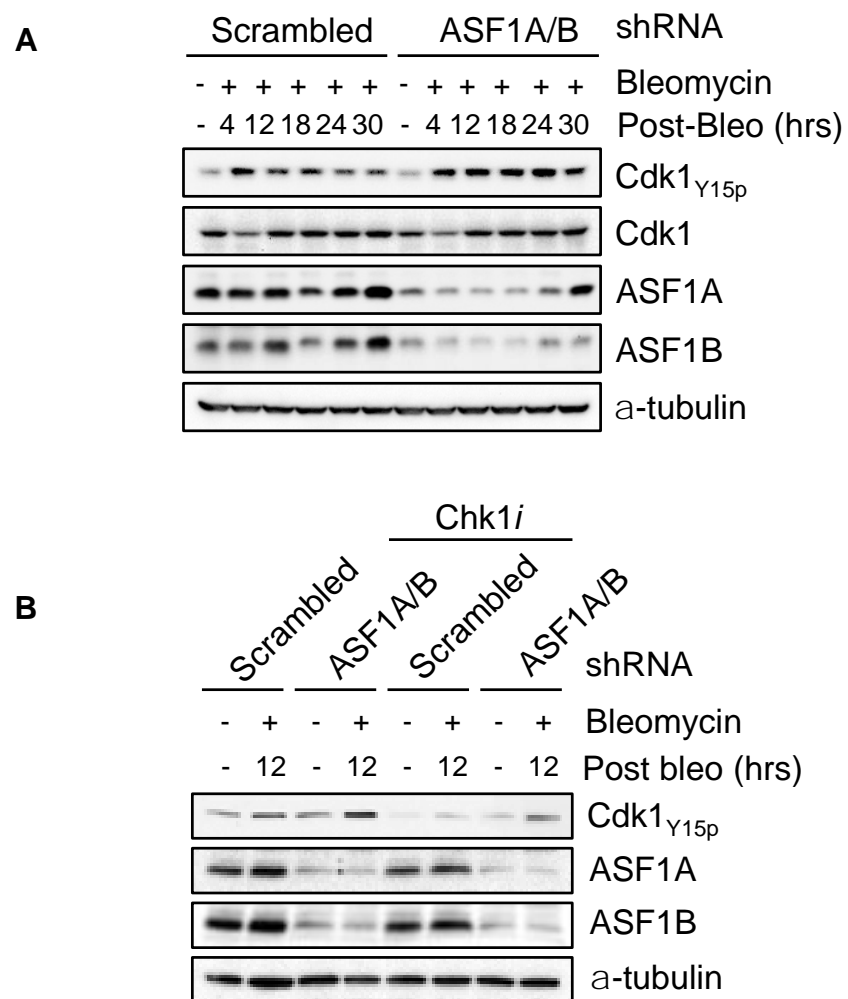
**Figure 4. ASF1A is required for both timely inactivation of Chk1 and efficient activation of Chk2. (A)** HeLa cells transduced with lentiviruses expressing scrambled shRNA or shRNA against both ASF1A and ASF1B for 72 hours were treated with 40  $\mu\text{g/ml}$  bleomycin for 2 hours, following by washing out the bleomycin at time 0. Samples were taken at the indicated time points after washing out the bleomycin, and western blotted for the indicated proteins or phosphorylation site. **(B)** Protein expression of ASF1A/B was silenced by siRNA-mediated knockdown for 48 hours, followed by bleomycin treatment as described in (A). **(C)** The phenotypes of DDR in ASF1 depleted cells are recapitulated after neocarzinostatin (NCS) treatment. To rule out drug specific effects, cells were incubated with another radiomimetic DNA damaging agent, NCS (0.5  $\mu\text{g/ml}$ ) for 2 hours followed by western blotting analysis. **(D)** Comparison of the turnover of Chk1 and Chk2 protein between control and ASF1 knockdown cells. Cells were treated with 50  $\mu\text{g/ml}$  Bleo for 2 hours. 10  $\mu\text{g/ml}$  cycloheximide (CHX) was applied for indicated time periods after Bleo removal. Protein levels of both Chk1 and Chk2 were detected;  $\gamma\text{H2AX}$  represents the existence of DNA breaks. **(E)** Complementation of ASF1A/B knockdowns with a plasmid expressing ASF1A is sufficient for the timely inactivation of Chk1. Knockdowns were performed as described in (A), the cells were transiently transduced with HA-tagged ASF1A<sup>WT</sup>. 24 hours after transfection, the experiment was performed as described in (A). **(F)** Examination of the effects of ASF1A and ASF1B alone on checkpoint kinase phosphorylation.

Next, I asked whether the persistent activation of Chk1 in the absence of ASF1 after DSB induction was accompanied by cell cycle arrest. Chk1 controls the G<sub>2</sub>/M cell cycle transition by regulating activities of downstream Cdc proteins, for example, Cdk1 phosphorylation at Tyr15 (Cdk1 Y15p) through mediating CDC25A/C degradation (Xiao et al., 2003). Cdk1 acts as critical gatekeeper to prevent premature entry into mitosis when it is phosphorylated in response to checkpoint activation (Shaltiel et al., 2015). Inducing DSBs in cells with ASF1 depletion led to persistent Cdk1 Y15p (**Fig. 5A**). Furthermore, the persistent phosphorylation of Cdk1 following DSB induction in ASF1 knockdown cells was Chk1 dependent, because it was abolished by addition of Chk1 inhibitor AZD7762 (**Fig. 5B**). In agreement with the persistent phosphorylation of Cdk1, DSB induction caused cells depleted of ASF1 to accumulate with a G<sub>2</sub>/M phase DNA content by flow cytometry (**Fig. 5C**), consistent with cell cycle arrest.

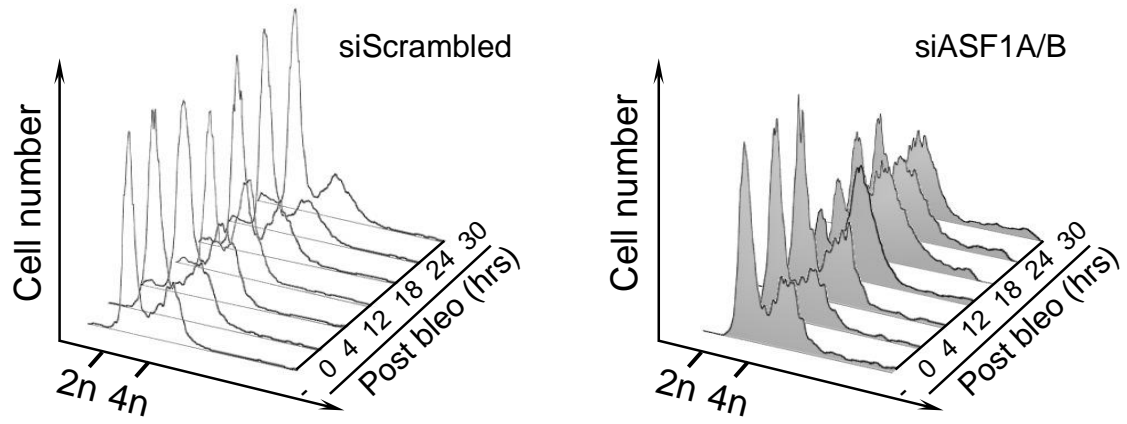
ASF1 is the upstream histone chaperone for replication-independent chromatin assembly of histones H3.3/H4 by the HIRA histone chaperone, and for replication-dependent chromatin assembly of histones H3.1/H4 by the CAF-1 histone chaperone (Hammond et al., 2017). To determine whether either of these chromatin assembly pathways were required for efficient inactivation of the ATR-Chk1 pathway after DSB induction, we knocked down HIRA and CAF-1. Inactivation of HIRA did not lead to persistent Chk1 phosphorylation and maintained comparable levels of Chk2<sub>T68p</sub> after DSB induction (**Fig. 5D**) indicating that the replication-independent chromatin assembly pathway is not

involved in inactivation of the ATR-Chk1 and ATM-Chk2 pathways. By contrast, knockdown of the large subunit of CAF-1, CAF-1 p150, led to persistent Chk1 phosphorylation, a greater reduction of Chk2 phosphorylation and persistent Cdk1 phosphorylation following DSB induction (**Fig. 5E, 3F**). Taken together, these data indicate that the replication-dependent chromatin assembly pathway plays a role in checkpoint regulation in humans, reminiscent of the situation in yeast (Chen et al., 2008).

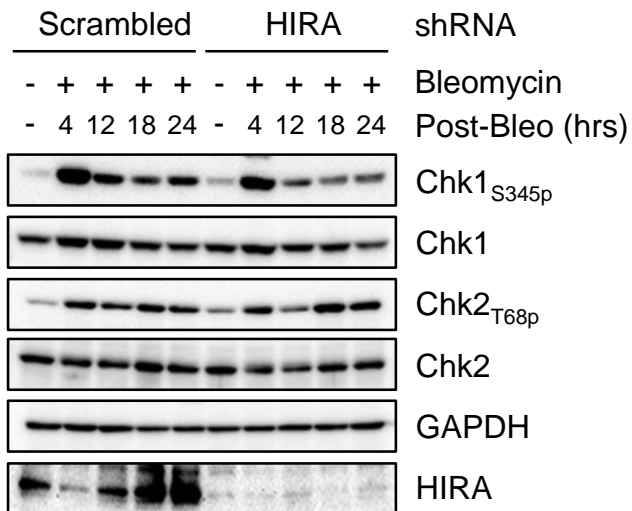
**Figure 5**



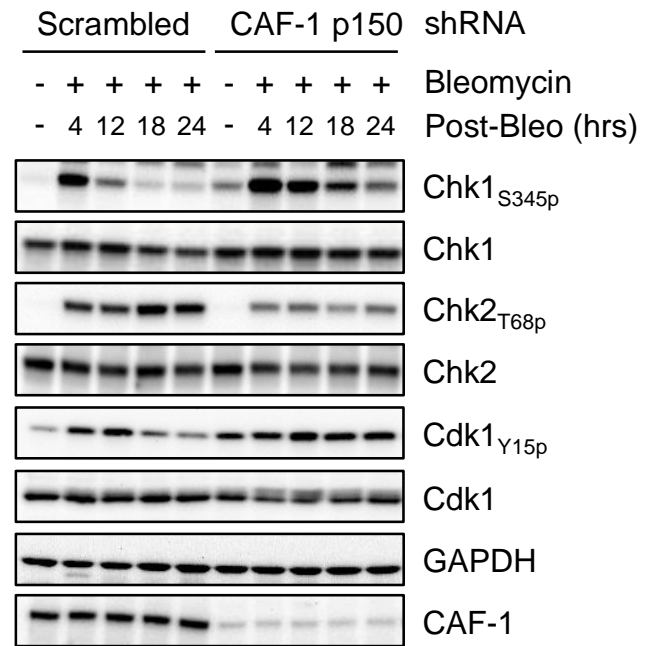
**C**



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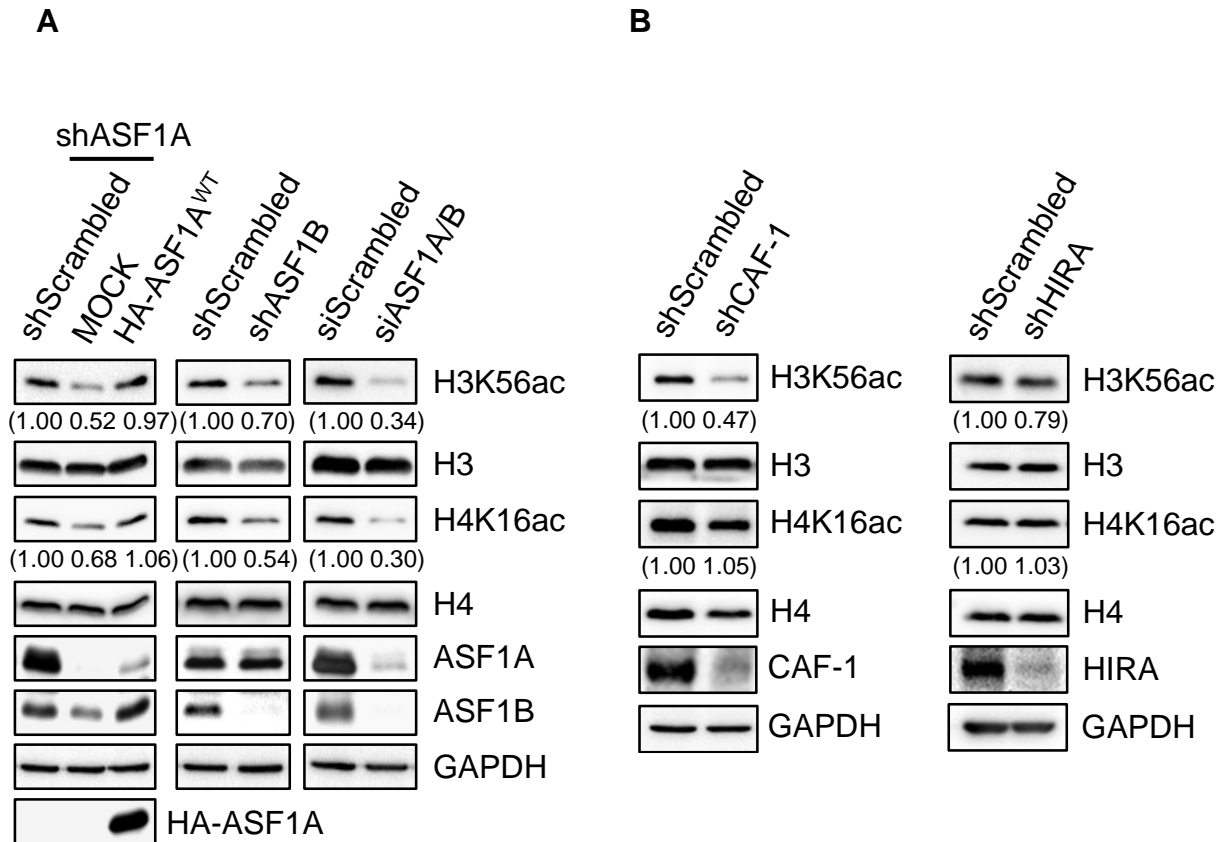
**Figure 5. Depletion of CAF-1, but not HIRA, phenocopies the dysregulated checkpoint kinases and the delay of cell cycle progression in ASF1 knockdown cells. (A) Robust Cdk1<sub>Y15p</sub> in the absence of ASF1A/B. The experiment was performed as described in Fig. 4A, with western blotting for the indicated proteins or modification. (B) Chk1 plays a major role in promoting Cdk1<sub>Y15</sub> phosphorylation in ASF1 depleted cells. 10  $\mu$ M of Chk1 inhibitor, AZD7762, was added to cells for an hour followed by co-incubating with bleomycin for an additional 2 hours. After bleomycin removal, 10  $\mu$ M of Chk1 inhibitor was re-applied for 12 hours. (C) ASF1-depleted cells accumulate in G<sub>2</sub>/M cell cycle phase while recovering from DSB damage, as shown by propidium iodide (PI) analysis of DNA content measure by flow cytometry. (D) HIRA depletion has no significant effect in affecting Chk1 and Chk2 phosphorylation. (E) CAF-1 depletion leads to persistent Chk1 and insufficient Chk2 activation accompanied with prolonged Cdk1 activation following DSB damage.**

### **3.2.2 Replication-dependent chromatin assembly affects the steady-state levels of acetylated H3 on Lysine 56 (H3K56ac) and H4 on Lysine 16 (H4K16ac) before and after DSB induction**

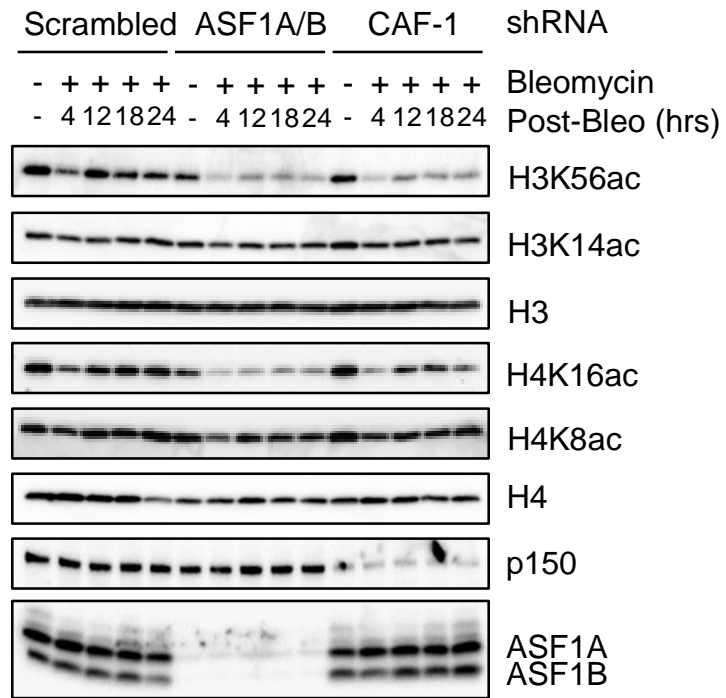
Replication-dependent chromatin assembly by ASF1 and CAF-1 is promoted by acetylation of histone H3 on K56, as this modification promotes the transfer of H3/H4 from Asf1 to CAF-1 in yeast. Acetylated H3K56 levels are rapidly reduced and reversed in response to DNA damage in human cells (Tjeertes et al., 2009). Therefore, while H3K56ac levels correlate with chromatin assembly, whether human ASF1 and other chaperones are involved in the recovery of H3K56ac after DSB repair remains unclear. I observed HeLa cells devoid of ASF1A or ASF1B partially reduced H3K56ac levels, which are further decreased upon double knockdown. Unexpectedly, I found H4K16ac levels were drastically decreased in the absence of ASF1, as compared to the control. The decreased acetylation of H3K56 and H4K16 can be reversed by the presence of shRNA-resistant ASF1A<sup>WT</sup> (**Figure 5A**). We also tested the effects of two ASF1 downstream histone chaperones, CAF-1 and HIRA, on acetylated H3K56 as well as H4K16. The western blotting analysis showed no significant changes in H4K16ac but the level of H3K56ac was markedly reduced in CAF-1-depleted cells whereas it is slightly affected by HIRA knockdown (**Fig. 6B**). These results showed that ASF1 and CAF-1 are required for maintaining the homeostasis of H3K56Ac possibly through newly-synthesized histone assembly. ASF1 alone may possibly affect H4K16ac via other unknown mechanism(s). To assess the change of acetylated H3 and H4 in response to DNA damage, I performed a time

course of post-radiomimetic drug treatment analysis of histone acetylation levels. Deacetylation of H3K56 and H4K16 started at 4-hr post-removal of bleomycin and the acetylation was mostly restored by 24 hours. However, the restoration of H3K56ac and H4K16ac were impeded in both ASF1A/B and CAF-1-depleted cells (**Fig. 6C**), but this was not the case in cells lacking HIRA even though they show slightly slower kinetics of recovery of acetylation (**Fig. 6D**). Other histone acetylations, such as H3K14ac and H4K8ac, were minimally affected, indicating H3K56ac and H4K16ac may specifically serve as histone markers for ASF1/CAF-1 mediated nucleosome incorporation.

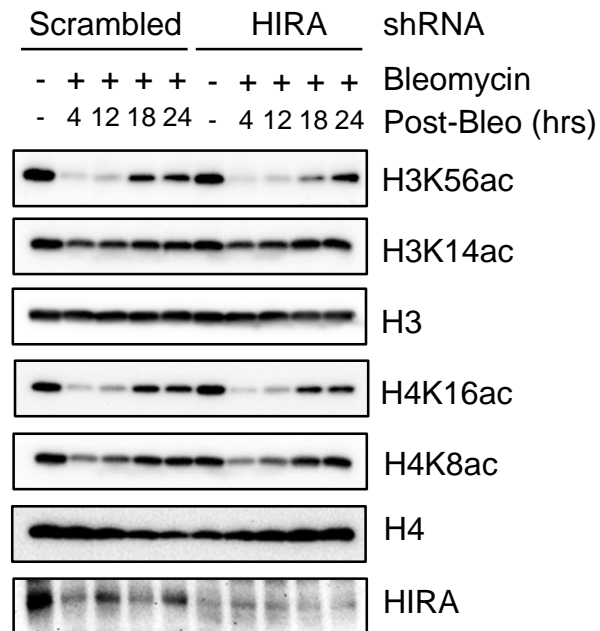
**Figure 6**



**C**



**D**

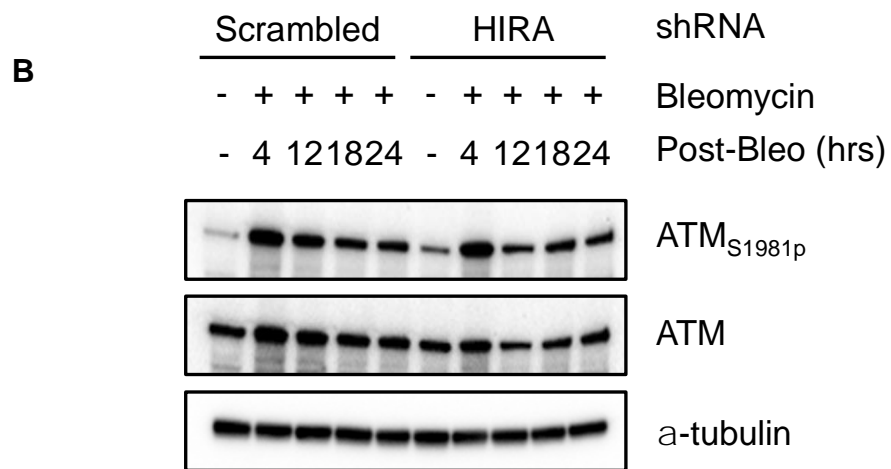
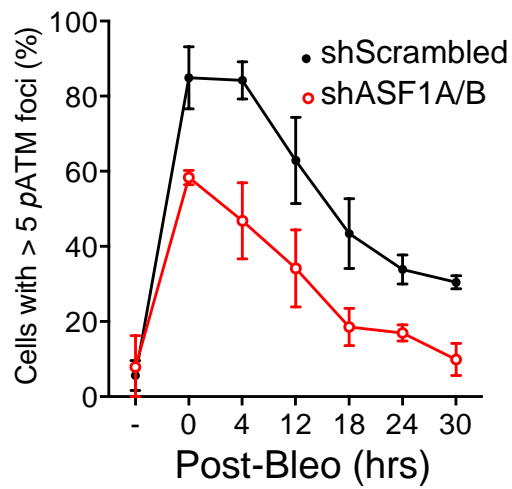
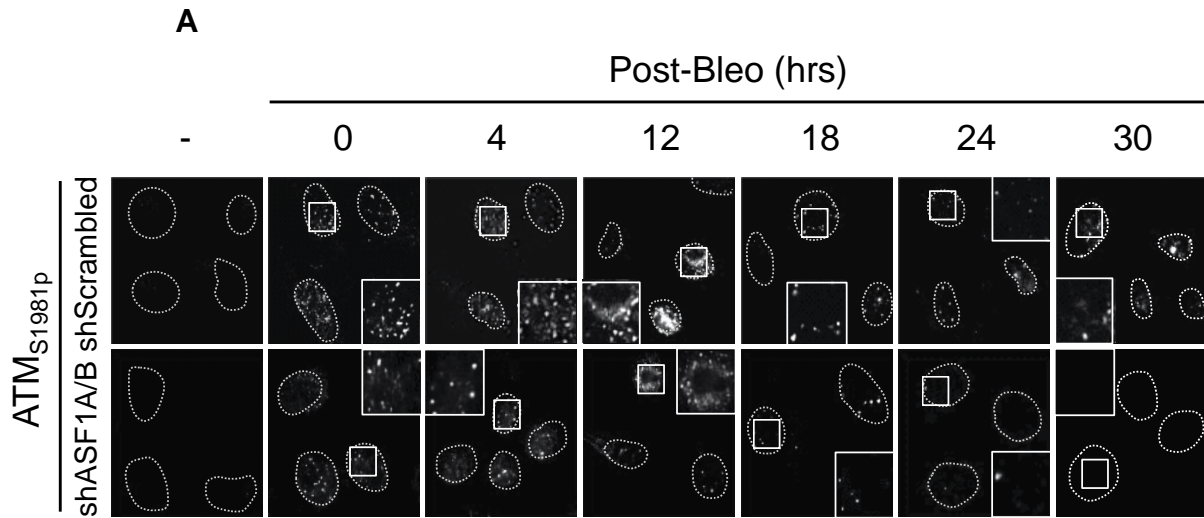


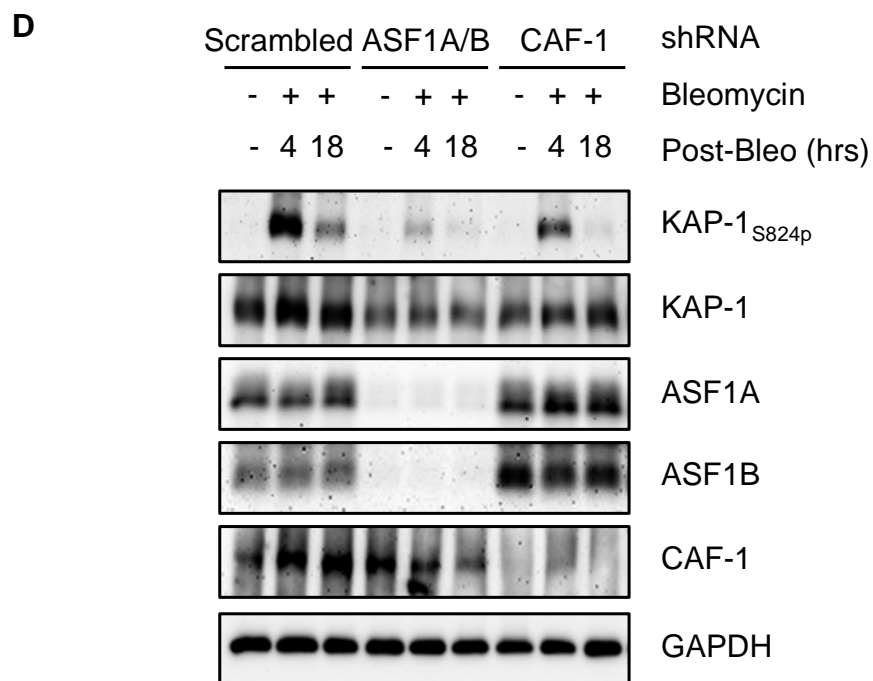
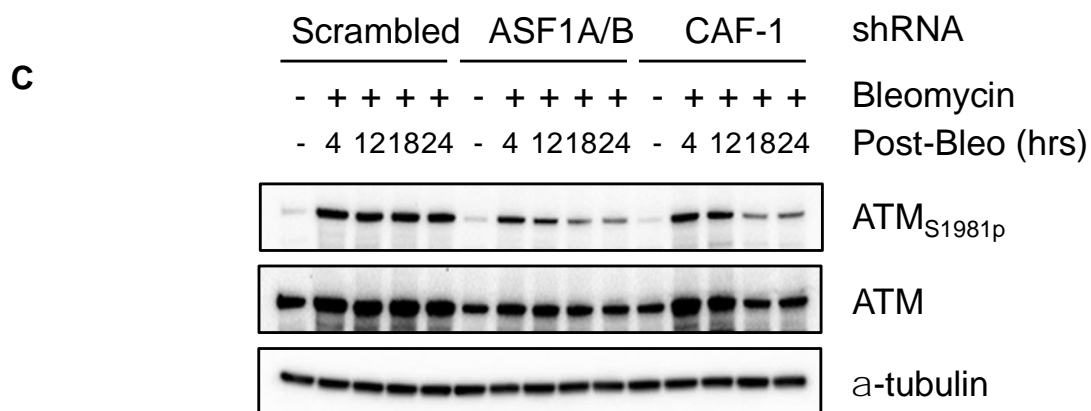
**Figure 6. ASF1 and CAF-1 mediate the homeostasis of H3K56ac and H4K16ac before and after DNA damage. Protein levels of H3K56ac and H4K16ac in the absence of either ASF1 or CAF-1 or HIRA. (A) ASF1 was silenced either by siRNA or shRNA. After 3-day transduction, ASF1A-depleted cells were transfected with shRNA resistant wild type ASF1A plasmid for 2 days. (B) CAF-1 and HIRA knockdown cells were collected and assayed by Western blotting 3 days post lentiviral transduction. Relative expression levels of H3K56ac and H4K16ac protein were normalized to H3 and H4, respectively. (C) (D) Histone chaperone-mediated recovery of H3 and H4 acetylation on specific residues after DNA damage. Cell with and without knockdown of either ASF1 or CAF-1 or HIRA were treated with 50  $\mu$ g/ml Bleo for 2 hours followed by various periods of incubation time after washing away the Bleo.**

### 3.2.3. ASF1/CAF-1 promote ATM activation and its nuclear localization

Based on the above observations, we sought to dissect the mechanism behind the phenotype from the DNA damage-signaling pathway. Given that ATM is known to be the primary kinase of Chk2 in humans, we wanted to understand whether ASF1 promotes ATM activation to affect signal transduction on Chk2 or other downstream factors. We also speculated that if this was the case, the recruitment of ATM to DNA damage sites may be mediated through histone chaperone-mediated alterations of the chromatin structure. Notably, DNA damage resulted in the recruitment of ATM<sub>S1981p</sub> into repair foci at DSB sites. ATM<sub>S1981p</sub> accumulation peaks within 4 hours after the removal of the DNA damaging agent and gradually declined over time (**Fig. 7A**). In contrast, even though the punctate ATM focus formation still occurs after the addition of bleomycin in ASF1-depleted cells, the number of ATM foci is much lower than control cells (**Fig. 7A**). Next, to assess whether ATM activity was also affected, the level of ATM phosphorylation on Serine 1981 which is a marker for its activity (Kozlov et al., 2006), was determined by western blotting. As expected, the levels of ATM<sub>S1981p</sub> upon ASF1 and CAF-1 silencing was reduced at 4 hours after the removal of bleomycin (**Fig. 7B**), whereas cells devoid of HIRA didn't give rise to a delay in boosting ATM activity (**Fig. 7C**). In addition, DNA damage-induced phosphorylation of KAP-1 (KAP-1<sub>S824p</sub>), which is directly mediated by ATM, was greatly reduced by knocking down ASF1 and CAF-1 (**Fig. 7D**). These results suggest that ASF1/CAF-1 contribute to efficient ATM activation and its localization to DSBs, leading to the reduced Chk2 and KAP-1 phosphorylation.

**Figure 7**





**Figure 7. ASF1 and CAF-1-mediated chromatin assembly contributes to the maintenance of long-lasting ATM kinase activity**

**(A) ASF1 promotes Bleo-induced ATM<sub>S1981p</sub> foci formation.** Following the recovery from 50  $\mu$ g/ml Bleo treatment for 2 hours, cells on coverslips were pre-extracted according to the immunostaining protocol described in



**Material and Methods.** Over 100 cells/ sample/ experiment were randomly picked to determine the number of foci in nuclei. Error bars were generated from 3 individual experiments. Effects on ATM<sub>S1981p</sub> protein levels responding to Bleomycin in the absence of (B) ASF1 and CAF-1 as well as (C) HIRA chaperones. (D) Loss of ASF1 and CAF-1 results in attenuated phosphorylation of KAP-1 after DNA damage.

#### **3.2.4. The replication-coupled chromatin assembly pathway promotes the transition from RPA-coated ssDNA to Rad51-coated ssDNA during homologous recombination.**

The persistent activation of Chk1 that occurs upon ASF1 and CAF-1 knockdown following DSB induction led us to ask if this is accompanied by persistent activation of the ATR kinase. By examining the kinetics of accumulation of ATR at DNA repair foci and dissipation of ATR from DNA repair foci at increasing times after washing out bleomycin, we observed persistent accumulation of ATR upon knockdown of ASF1, as compared to the control (**Fig. 8A**). Consistent with ATR activation by RPA bound to ssDNA, we also observed persistent retention of RPA at DNA repair foci at increasing times after washing out bleomycin upon ASF1 knockdown, as compared to the control but not in the absence of bleomycin (**Fig. 8A**). RPA is phosphorylated by multiple different kinases on numerous residues of its RPA2 subunit in response to DSBs resected to form ssDNA (Marechal and Zou, 2015). In this RPA phosphorylation cascade, phosphorylation of Ser4/Ser8 by DNA-PKcs appears to be the final event in the

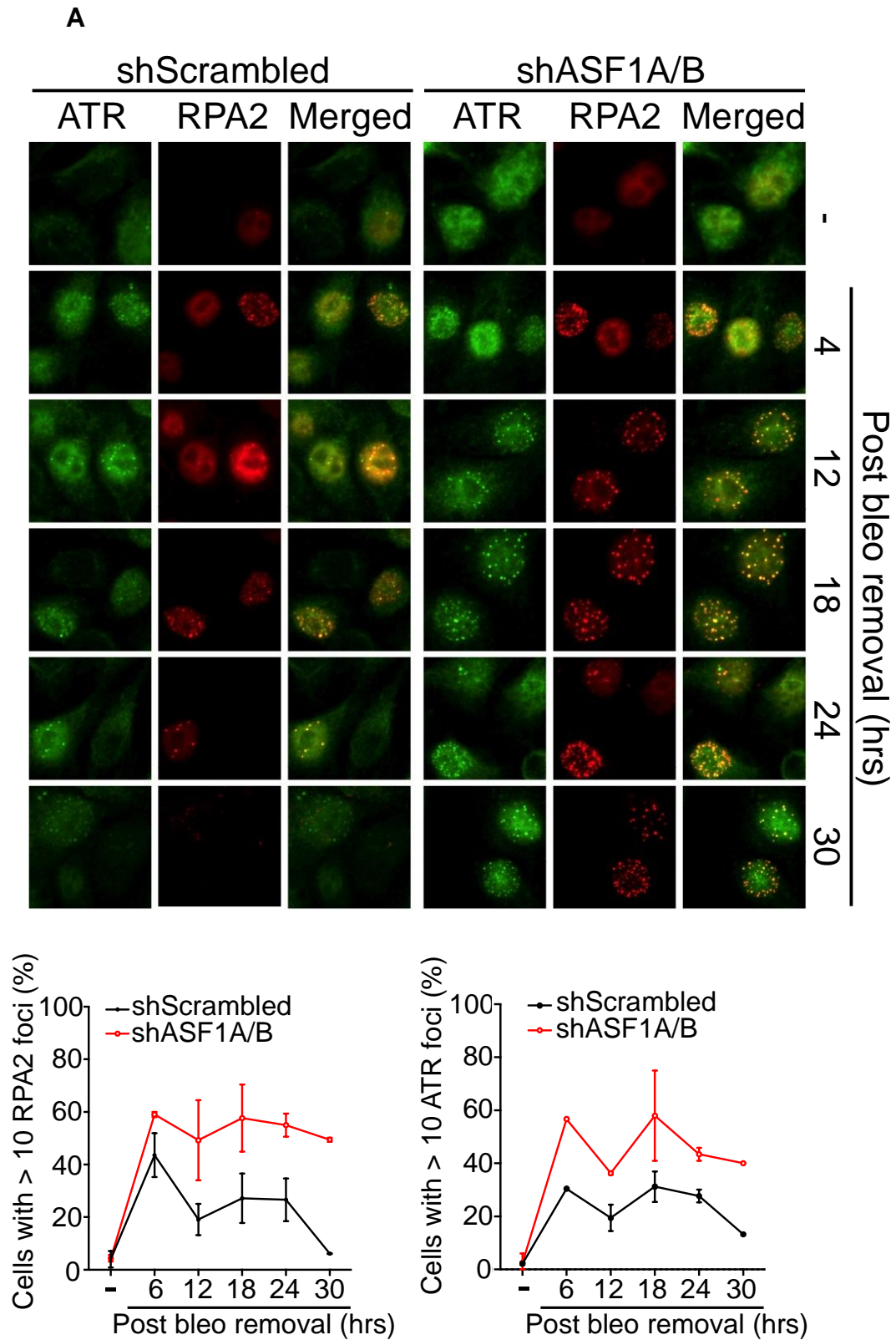
maturation of DNA damage-induced hyperphosphorylated RPA, where these phosphorylations specifically delay mitotic entry (Liaw et al., 2011). Furthermore, Ser4/Ser8 phosphorylation of RPA2 is important for full ATR activation (Liu et al., 2012). Consistent with there being persistent DSBs resected to ssDNA in cells depleted of ASF1A/B, we saw persistent Ser4/Ser8 phosphorylation on RPA2 (**Fig. 8B**) but only after inducing DSBs. While the persistent Chk1 phosphorylation seen in ASF1 depleted cells after DSB damage was blocked by the ATR inhibitor, VE-821 this was not the case with the RPA2 Ser4/Ser8 phosphorylation (**Fig. 8C**), consistent with it being mediated by DNA-PKcs. The persistently activated RPA2 was also observed upon CAF-1 knockdown cells, but not upon HIRA knockdown (**Fig. 8D**), indicating that the replication-dependent chromatin assembly pathway was required for terminating hyperactivated RPA.

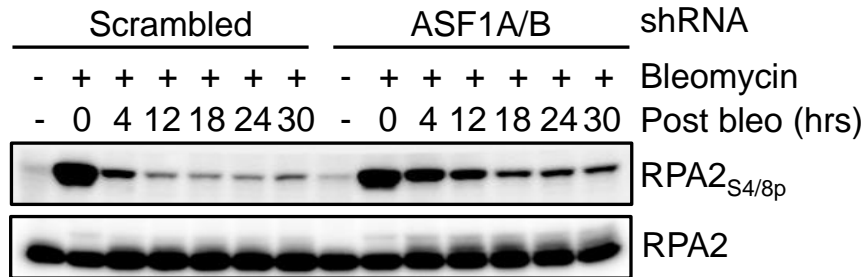
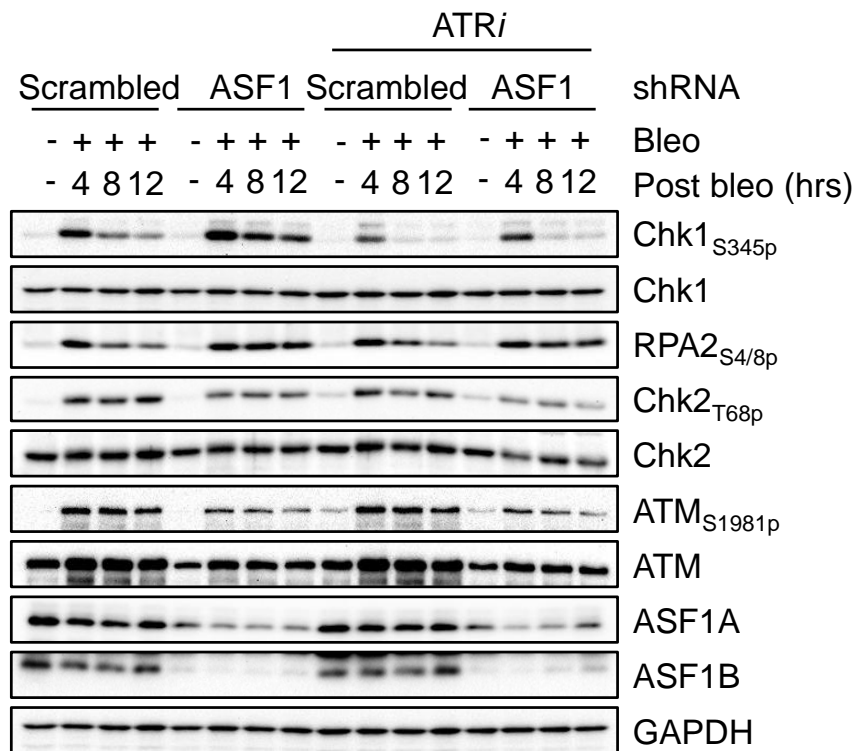
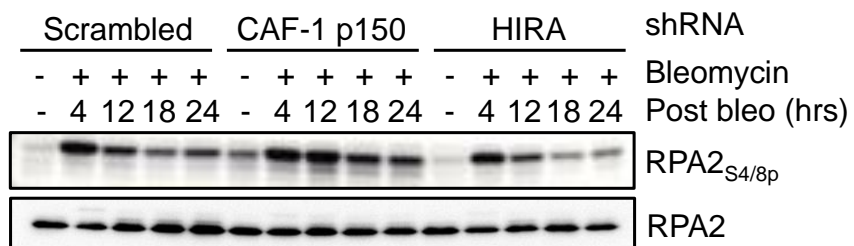
To understand the mechanism behind the prolonged ATR-RPA2-Chk1 signalling upon defective replication-dependent chromatin assembly, we asked if this was due to there being more ssDNA present after DSB induction in ASF1 depleted cells. We used native BrdU immunofluorescence staining to detect BrdU in ssDNA but not within dsDNA (Raderschall et al., 1999) after exposure of cells to camptothecin or bleomycin. Camptothecin is a topoisomerase I poison that induces DSBs during replication. The amount of BrdU at ssDNA upon depletion of ASF1 was significantly higher than in the control cells following camptothecin or bleomycin treatment (**Fig. 8E**). As a control for reduced end resection during DSB repair, we knocked down the exonuclease CtIP that

mediates DNA resection (Sartori et al., 2007), and as a control for a situation where all DSBs would undergo DNA resection, we knocked down the resection blocking protein 53BP1 (Bothmer et al., 2010). Noteworthy, the amount of end resection observed upon ASF1 knockdown was comparable to the extreme amount of resection that occurs upon 53BP1 knockdown. These results suggest that ASF1 plays a role in protecting DSB ends from excessive resection.

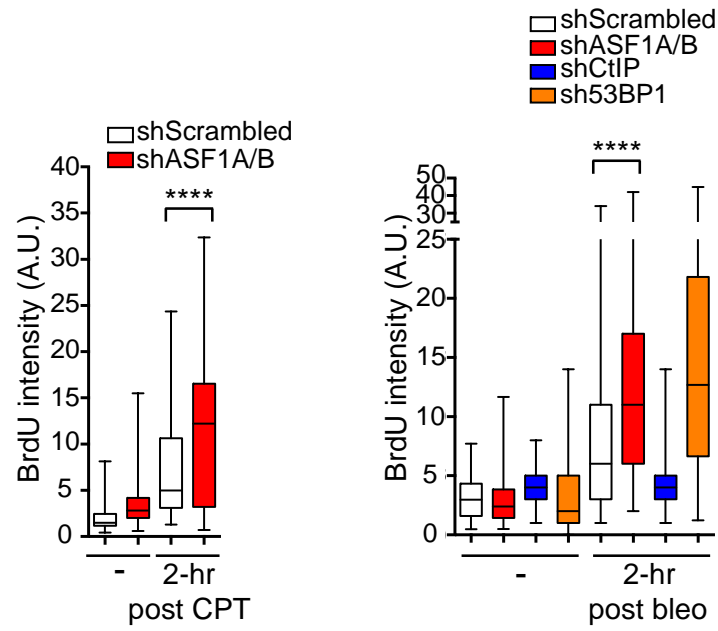
During the process of homologous recombination, RPA is displaced from ssDNA by an unclear mechanism, and is replaced by Rad51 to form the nucleofilament that initiates strand invasion. Given that there was persistent RPA activity and foci formation DSB induction in ASF1 depleted cells, we asked if this was accompanied by reduced Rad51 nucleofilament formation. Compared to control cells, we observed a significant reduction in the number of cells with Rad51 foci after DSB induction in ASF1 depleted cells (**Fig. 8F**). Consistent with the reduced recruitment of Rad51 upon ASF1 depletion, we observed a significant defect in homologous recombination upon ASF1 depletion using a chromosomally integrated DR-GFP reporter in U2OS cells that allows homologous gene conversion induced by the I-SceI nuclease (Pierce et al., 1999) (**Fig. 8G**). Taken together, the persistent RPA2 foci accumulation and extensive DNA resection, coupled with the reduced Rad51 loading and impaired homology gene conversion in the absence of ASF1 after DSB induction, reveal an important new function of ASF1 in HR.

Figure 8

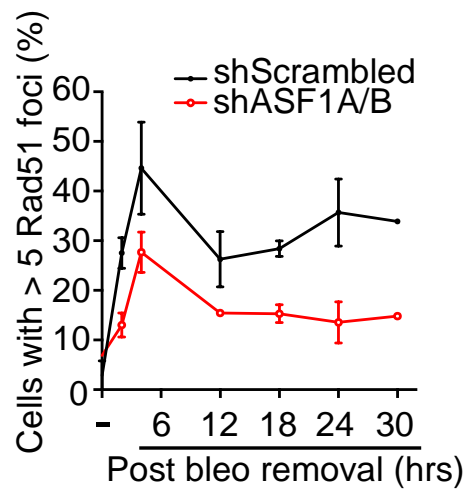
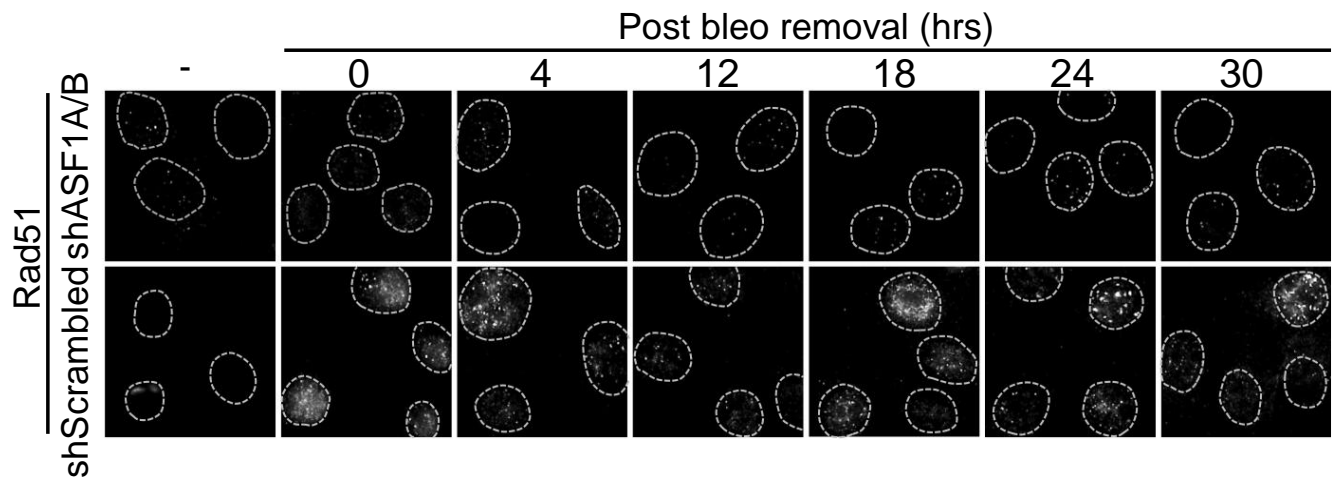


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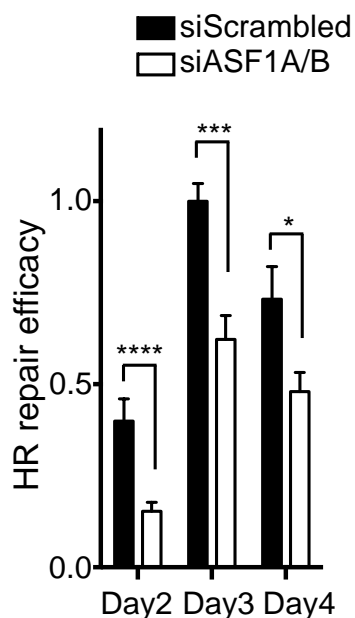
**E**



**F**



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**Figure 8. ASF1 is required for the efficient replacement of RPA with Rad51 and promotes DNA homologous recombination repair. (A) Prolonged ATR and RPA2 foci accumulation in the absence of ASF1 following DSB damage. Cells were treated as described in Fig. 4A. Quantitation of RPA or ATR foci is shown below, measured from > 100 cells from three independent experiments, with average and standard deviation plotted. (B) Robust RPA2 phosphorylation in ASF1 depleted cells following DSB damage. The experiment was performed as described in Fig. 4A. (C) Robust RPA2 phosphorylation in CAF-1 depleted cells, but not HIRA depleted cells, following DSB damage. (D) Accumulation of ssDNA in ASF1 depleted cells after DSB damage. BrdU fluorescence intensity in nuclei is depicted by**

box-and-whisker plot showing medians (lines in boxes) of  $n > 100$ , 25<sup>th</sup>-75<sup>th</sup> percentiles (boxes) and overall ranges. Student's t-test: \*\*\*\* $P < 0.0001$ . (E) ASF1 depleted cells are defective in Rad51 nucleofilament formation. Cells were treated as described in Fig. 4A. Dashed outlines represent nuclei based on DAPI staining. Quantitation is shown based on the average and standard deviation from three independent experiments. (F) ASF1 depletion reduces HR efficiency. The frequency of GFP-positive cells measured by flow cytometry at 24 hours, 48 hours and 72 hours after I-SceI transduction in DR-GFP U2-OS cells is shown. Error bars indicate S.E.M.;  $n=3$ . t-test: \*\*\*\* $P < 0.0001$ , \*\*\* $P = 0.0007$ , \* $P = 0.0118$ .



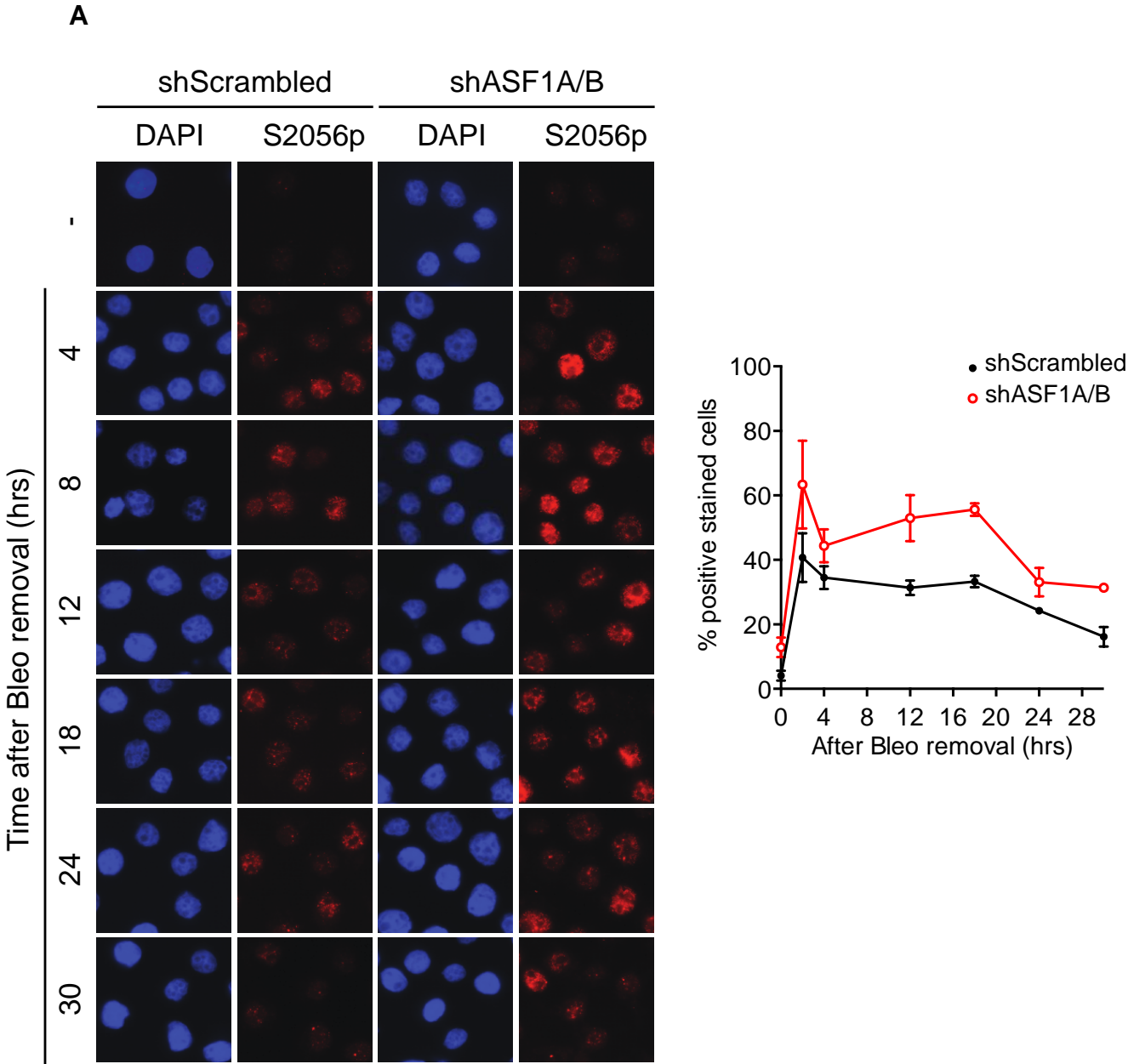
### 3.2.5. ASF1 restrains DNA-PKcs autophosphorylation and its accumulation on chromatin after DSB induction

In terms of the elevated RPA2<sub>S4/8p</sub> level in bleomycin-challenged ASF1 knockdown cells, it was of interest to understand whether ASF1 affects RPA2<sub>S4/8p</sub> through the upstream kinase, DNA-PKcs. When cells were challenged with bleomycin, DNA-PKcs<sub>S2056p</sub> accumulated at DNA repair foci and gradually disappeared over time during recovery from DNA lesions (**Fig. 9A**). Conversely, despite the kinetics of DNA-PKcs focus disappearance in ASF1 knockdown cells being comparable to what was seen in the control, the DNA-PKcs staining became more intense accompanied with the elevated number of “+” ve stained cells with the devoid of ASF1 (**Fig. 9A**). This result was confirmed through flow cytometry against phospho DNA-PK protein, showing that Bleo-treated ASF1-depleted cells from 6 hours during recovery had a 2-fold increase in DNA-PK “+” ve stained population as compared to the control (**Fig. 9B**). The specificity for the antibody against DNA-PKcs<sub>S2056p</sub> and DNA-PKcs<sub>T2609</sub> was confirmed upon DNA-PK knockdown by western blotting (**Fig. 9C**). In addition, I also observed robust level of DNA-PKcs autophosphorylation at serine 2056 in ASF1 knockdown cells (**Fig. 9C**), which was consistent with the prolonged RPA2 s4/8 phosphorylation. Next, given that DNA-PKcs is pivotal in NHEJ repair, we tested the impact of ASF1 on the end joining repair process. The I-SceI-mediated GFP reporter system in which GFP was expressed after the completion of DNA repair via the NHEJ pathway was used to determine repair efficiency (Mao et al., 2008). Following I-SceI constitutive expression, the GFP signal in ASF1-depleted cells

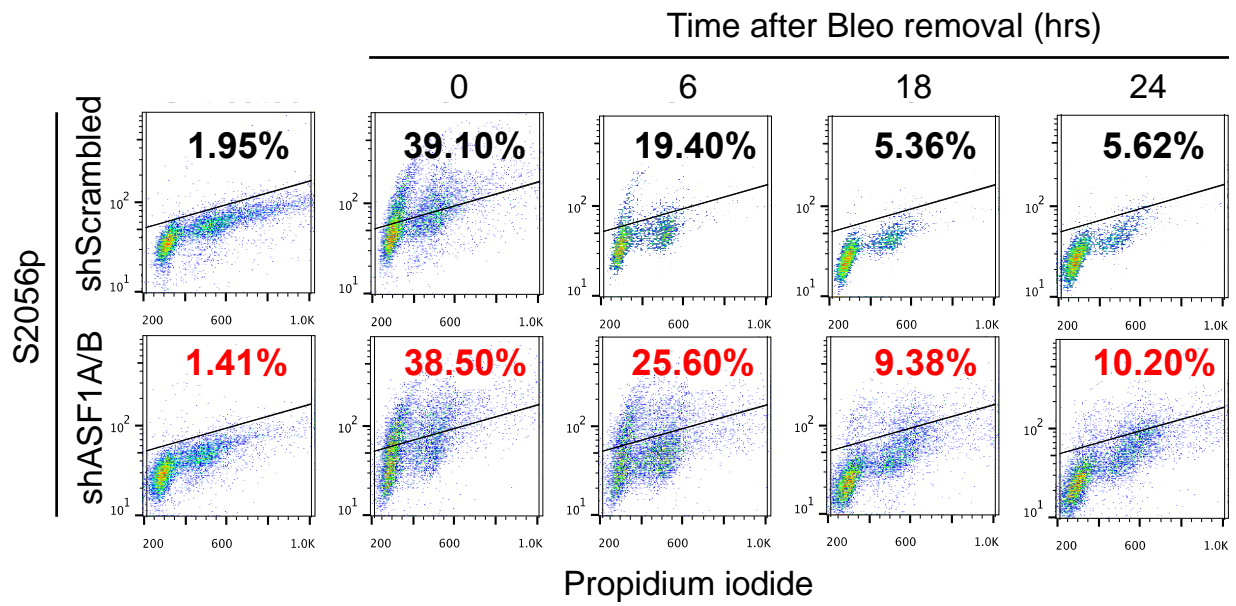
measured by flow cytometry was similar to control cells at Day 3 but was higher than control cells at Day 4 (**Fig. 9D**). Next, the inducible DSB cutting / repair system driven by I-Ppol endonuclease was used to compare DNA repair kinetics between control and ASF1 depleted cells after DSB induction. We have previously shown this inducible DSB system is mainly repaired by NHEJ (Li and Tyler, 2016). By real-time PCR analysis with primers flanking the I-Ppol cutting site, the ratio of DNA cutting at SLCO5A locus was achieved to ~ 50% which was defined as “0” time point right after washing off 4-OHT and Shield-1 (**Fig. 9E**). With increasing time of recovery, the I-Ppol-induced DSB was nearly completely repaired in 6 hours (**Fig. 9E**). When ASF1A was knocked down in HCT116 cells, the kinetics of DNA repair matched that which was observed in control cells, indicating that ASF1 does not affect NHEJ-mediated DSB repair in the I-Ppol system (**Fig. 9E**). By taking advantage of inducible site-specific breaks, I was able to validate the impact of ASF1 on the extent of DNA-PKcs (and other factors) accumulation on chromatin. Chromatin immunoprecipitation (ChIP) was carried out with phospho-DNA-PKcs<sup>S2056</sup> antibodies and the level of target chromatin near the I-Ppol cutting site was quantitated by real-time PCR. Indeed, DNA-PKcs<sup>S2056p</sup> was recruited in the presence of DNA breaks and gradually disassociated during DNA repair (**Fig. 6F**). However, depletion of ASF1A significantly elevated DNA-PKcs mobilization to chromatin adjacent to the break site at each time point following DSB induction but still caused the dissipation of DNA-PKcs after completion of DNA repair (**Fig. 6F**). Similar to DNA-PKcs, XRCC4, an essential component for DNA ligation during NHEJ was drastically

enriched at DSBs in the absence of ASF1A (**Fig. 6G**). Taken together, these results suggest that ASF1A is required for promoting the timely dissociation of NHEJ machinery from repaired chromatin.

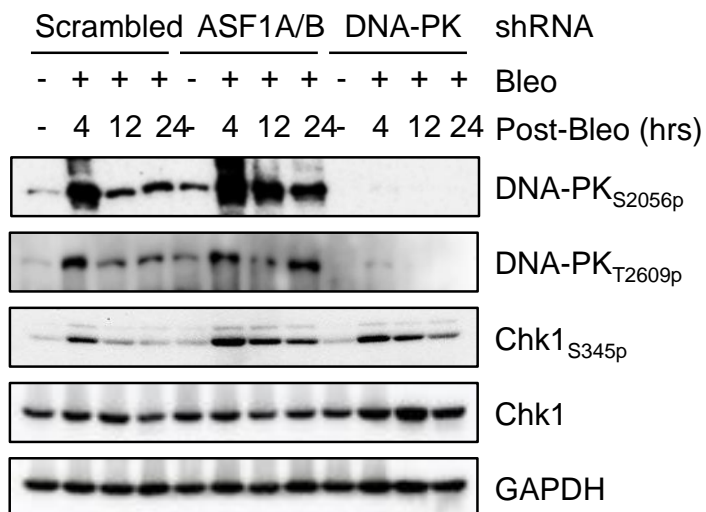
**Figure 9**



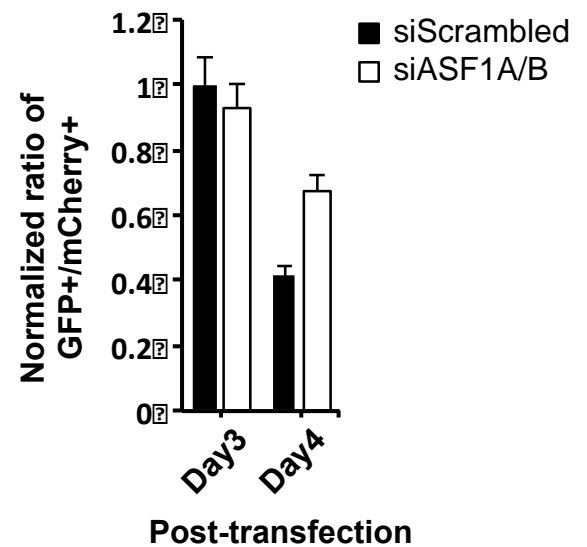
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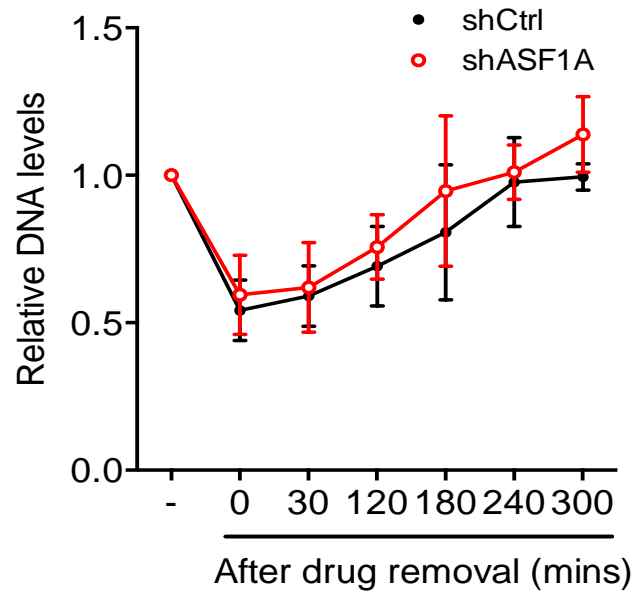
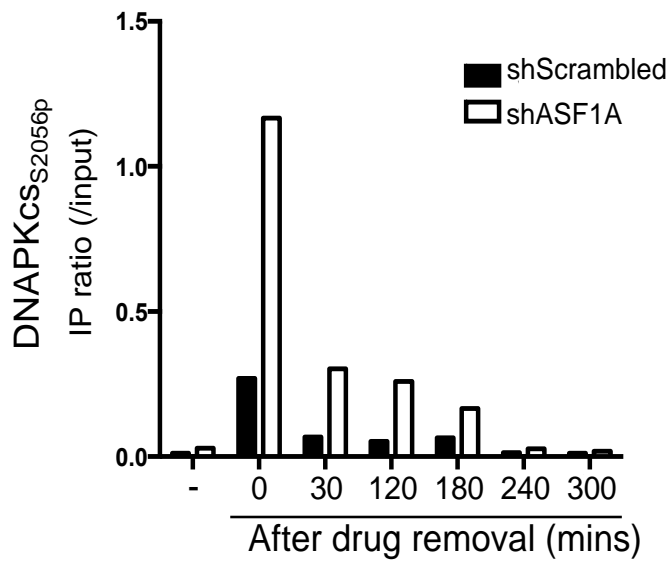
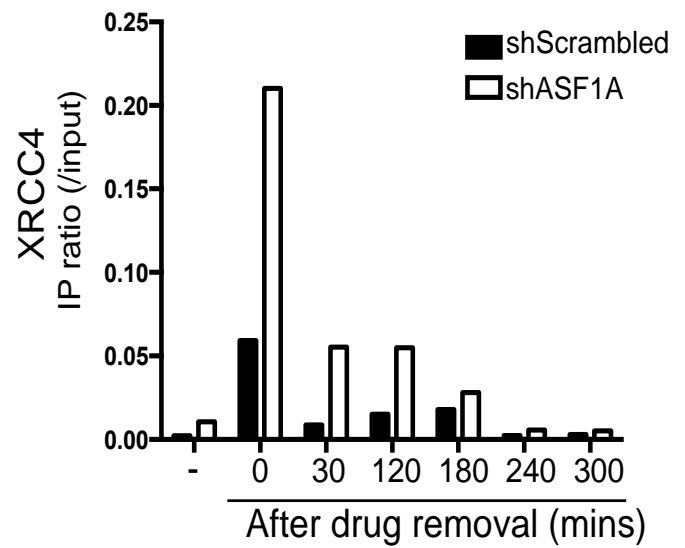


**C**



**D**



**E****F****G**

**Figure 9. ASF1 depletion promotes accumulation of the NHEJ complex on chromatin at DSB sites. (A) ASF1 negatively regulates the DNA-PKcs<sub>S2056p</sub> foci formation in the presence of DNA damage. After exposure to Bleo, cells were harvested for immunostaining analysis. The percentage of DNAPK<sub>S2056p</sub> +ve cells was calculated from over 100 cells / sample / experiments (n=3). (B) ASF1 depletion leads to an increased population of DNAPK<sub>S2056p</sub> + ve cells over time compared to control cells challenged by 50 µg/ml Bleomycin. Cells were harvested at indicated time points followed by 70% ethanol fixation. Fixed cells were blocked with 3% BSA for 1 hour and incubated with the DNAPK<sub>S2056p</sub> antibody for an additional hour. After conjugated with fluorescent secondary antibody and propidium iodide, cells were analyzed by flow cytometry. The percentage of DNAPK<sub>S2056p</sub> +ve cells is shown in each panel. (C) The level of DNA-PKcs phosphorylation upon ASF1 knockdown was elevated in response to DNA damage. (D) U2-OS cells stably integrated with the I-sceI-GFP-based NHEJ reporter were transduced with 100 nM of both ASF1A and ASF1B siRNAs for 48 hours followed by co-transfection with constitutive expressed I-SceI and mCherry plasmid for 3 and 4 days. After flow analysis, the value of GFP+ was normalized to mCherry+ as the DNA repair ratio. (E) Impact of ASF1A on NHEJ-mediated DNA repair. The dd-IPpol-HCT116 stable line was transiently transduced with either shScrambled or shASF1A lentivirus for 72 hours. After the addition of 10 µM Shield-1 for 3 hours, cells were harvested at the indicated time points. Real-time PCR values were**

normalized to GAPDH in each sample. Error bars were obtained from three individual experiments. (F)(G) Impact of ASF1A on the site-specific DSB induced-enrichment of DNAPKcs<sub>S2056p</sub> and XRCC4 on chromatin. 2 µg/ml of DNAPK<sub>S2056p</sub> and 5 µg/ml XRCC4 antibodies were used for ChIP analysis, respectively, and data were normalized to DNA input.

### **3.2.6. Loss of MMS22L phenocopies loss of ASF1 and CAF-1 during homologous recombination.**

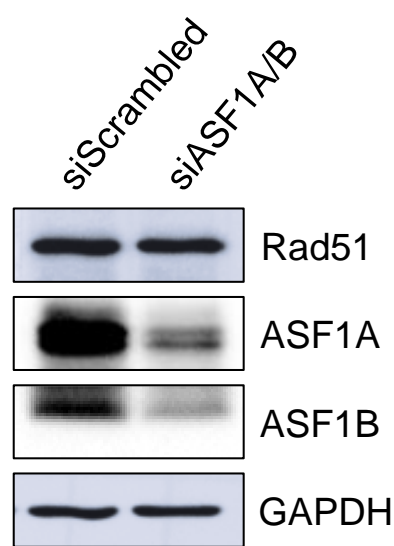
Reduced Rad51 loading onto ssDNA in the ASF1 depleted cells could be for many reasons. However, this was not an indirect consequence of reduced levels of total Rad51 protein in ASF1 depleted cells, because the Rad51 protein levels were not changed by ASF1 knock down (**Fig. 10A**). As discussed above, MMS22L/TONSL promotes Rad51 nucleofilament formation (Duro et al., 2010; O'Donnell et al., 2010; Piwko et al., 2016). Given that ASF1 also promoted Rad51 nucleofilament formation (**Fig. 8F**) and that ASF1A/B interact with MMS22L/TONSL (Duro et al., 2010) we investigated the potential of MMS22L/TONSL and ASF1 functioning in the same pathway to promote Rad51 loading onto resected DNA during HR. Reminiscent of what we observed with cells depleted of ASF1 or CAF-1, MMS22L depletion also led to persistent phosphorylation of Chk1<sub>S345p</sub>, RPA2<sub>S4/S8p</sub> and Cdk1<sub>Y15p</sub>, following removal of bleomycin (**Fig. 10B, 7C**). Like ASF1 depletion, CAF-1 depletion and MMS22L depletion also led to less Rad51 foci after removal of bleomycin (**Fig. 10D**). As a physical measure of a DNA repair defect, we performed comet assays. For these analyses, we used camptothecin (CPT) because it enriches for DSBs that are repaired by homologous recombination. Following DSB induction, cells depleted of ASF1, CAF-1, MMS22L or Rad51 all had long tail moments that were not statistically different from each other, but were significantly longer than the control, suggesting that loss of ASF1, CAF-1, MMS22L or RAD51 causes a similar degree of defect in homologous recombinational repair (**Fig. 10E**). Taken



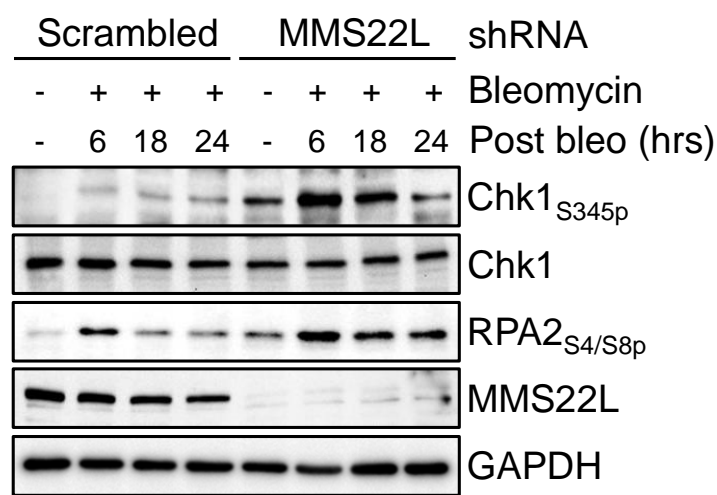
together, these results suggest that MMS22L and the H3.1/H4 chaperones likely function in the same pathway to promote Rad51 loading onto resected DNA during DSB repair.

Figure 10

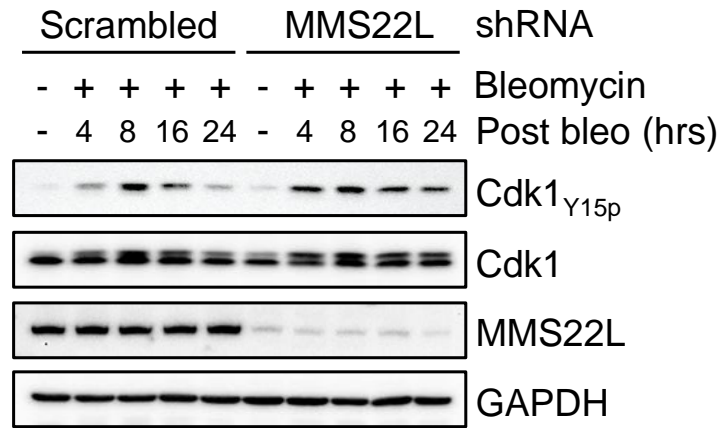
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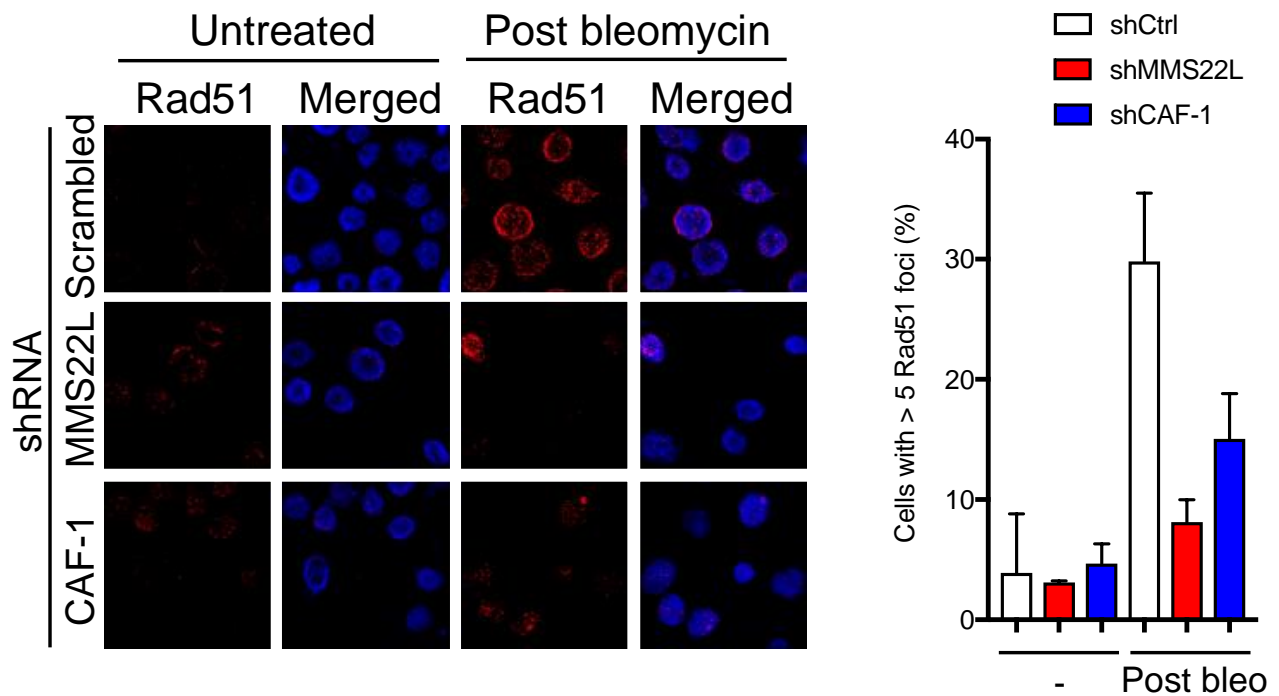
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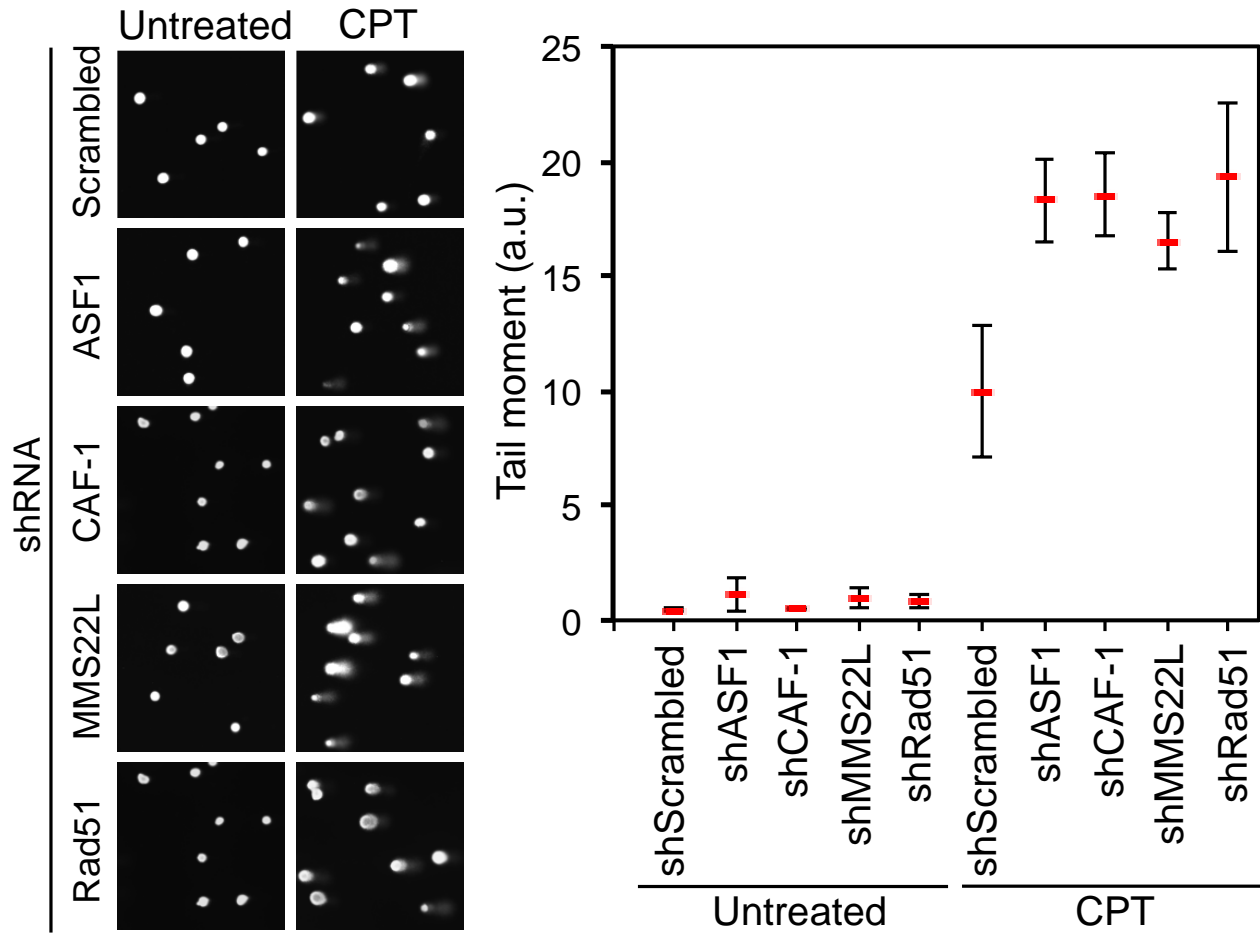
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E



**Figure 10. MMS22L depletion phenocopies depletion of ASF1 or CAF-1. (A)** Comparison of Rad51 protein expression between control and ASF1 knockdown cells. Scrambled and ASF1A/B knockdowns were preformed with siRNA for 48 hours. **(B)** DNA damage-induced hyper-phosphorylation of Chk1 and RPA2 **(B)** and Cdk1 **(C)** following MMS22L depletion. The experiment was performed as described in Fig. 4-5 but with depletion of

**MMS22L. (D) Depletion of CAF1 p150 or MMS22L attenuated Rad51 focus formation 24 hours after bleomycin removal. Shown is the quantitation from over 100 cells per sample per experiment from three independent experiments, with the average and standard deviation plotted. (E) Increased tail moments upon ASF1, CAF-1 and MMS22L depleted cells following DSB exposure. K562 cells were incubated with 10  $\mu$ M CPT for 2 hours followed by alkaline comet assay. Comet tail moments were calculated by CaspLab comet assay software for > 60 cells in each sample. The red line represents the mean value of tail moment shown in the panel; error bars were obtained from two individual experiments.**

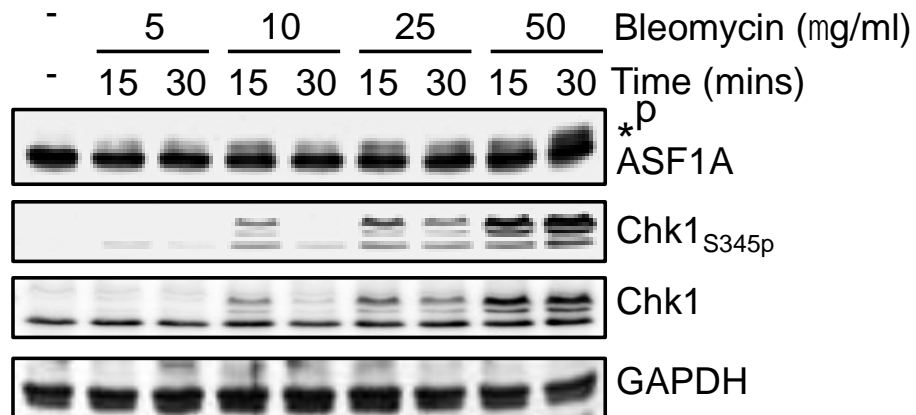
### 3.2.7. DNA-PKcs phosphorylates ASF1A at serine 192 in response to DNA damage

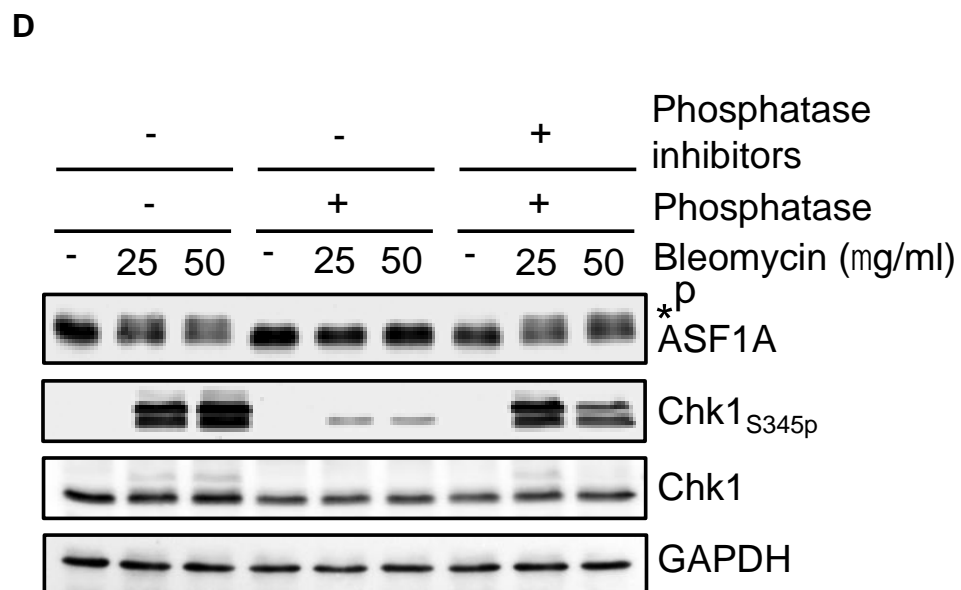
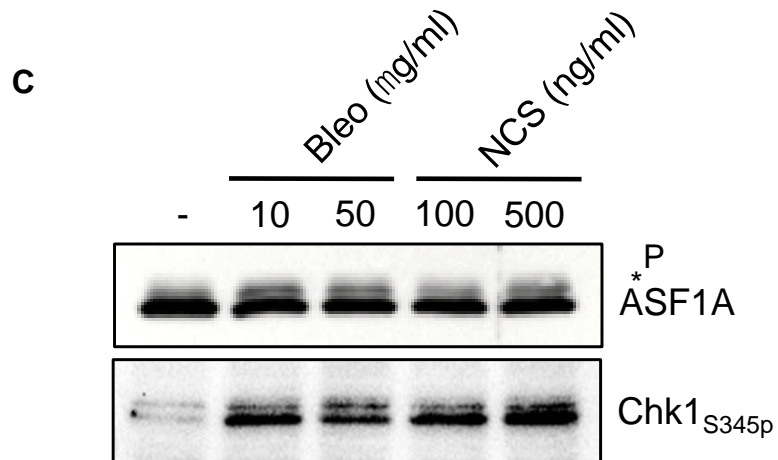
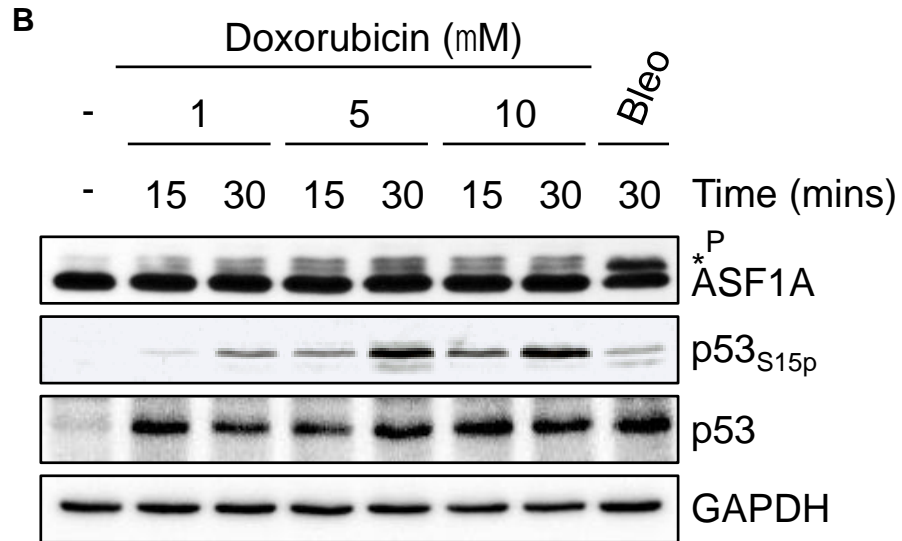
During the course of our analyses, I consistently observed a slower migrating form of ASF1A appearing within 15 minutes after treatment with the DSB inducing agents bleomycin and doxorubicin in a dose responsive manner (**Fig. 11A, Fig. 11B**). This phenomenon was also observed in MEF cells treating with bleomycin and neocarzinostatin (**Fig. 11C**). This represents phosphorylated ASF1A, because the slower migrating form was abolished by treatment with phosphatase (**Fig. 11D**). ASF1A is known to be phosphorylated by Tausled-like kinases (Tlks) (Mello et al., 2002). However Tlks are specifically inhibited by ATM in response to DNA damage (Groth et al., 2003), indicating that the damage-induced phosphorylation of ASF1A is performed by kinases other than Tlks. In agreement, we observed that the damage-induced phosphorylation of ASF1A occurs concomitant with the damage-induced loss of phosphorylation of the Tlks substrate Rad9 S328 (Sunavala-Dossabhoy and De Benedetti, 2009) (**Fig. 11E**). Therefore, we tested whether the DDR kinases were involved and found that inhibition of neither ATM nor ATR prevented the DSB-induced phosphorylation of ASF1A (**Fig. 11F,G**). However, inhibition of DNA-PKcs with either NU-7026 or NU-7441 significantly reduced the damage induced ASF1A phosphorylation (**Fig. 11H, I**). As positive controls for DNA-PK inhibition, we examined the DNA-PK substrate RPA Ser4/8p and Chk1 S345p, given that DNA-PK is known to prime ATR via its role in RPA phosphorylation (Vidal-Eychenie et al., 2013). ShRNA knockdown of DNA-PK also reduced damage-induced ASF1A phosphorylation

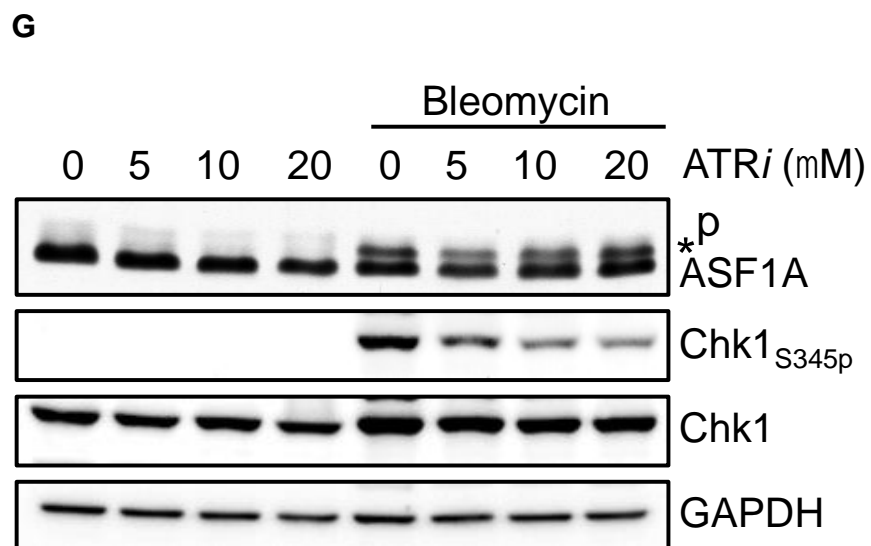
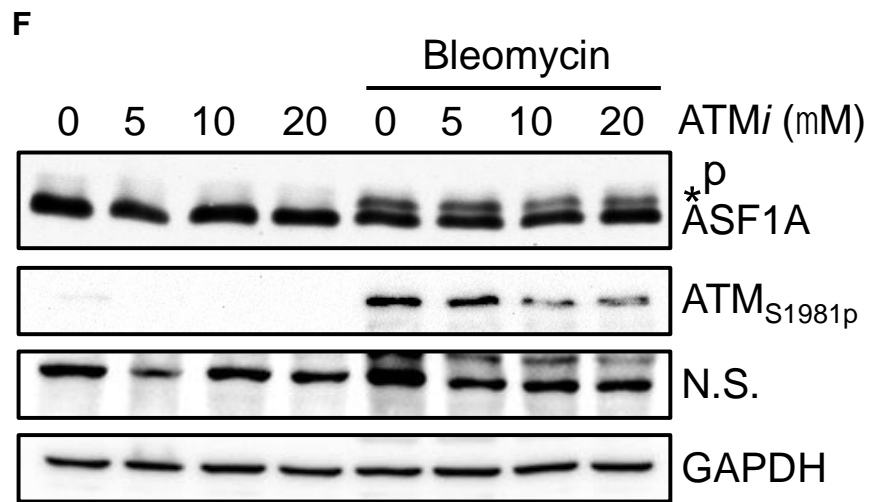
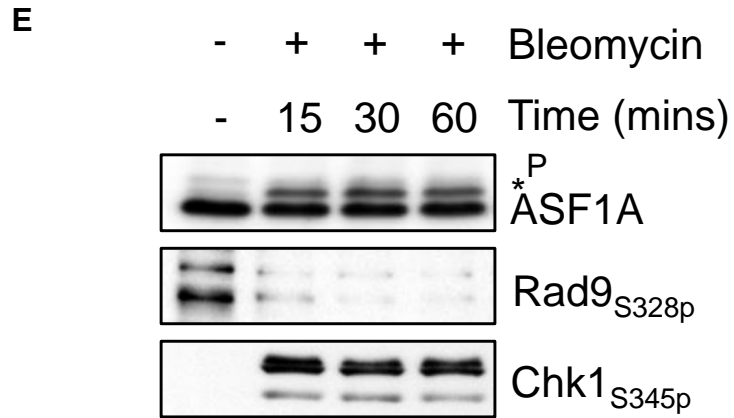
(**Fig. 11J**) in a manner proportional to the efficiency of the knockdown. Finally, damage-induced ASF1A phosphorylation failed to occur in M059J human glioblastoma cells that have a frameshift in the gene encoding DNA-PKcs preventing DNA-PK expression (Allalunis-Turner et al., 1993), while the M059K cell line made from another region of the same tumor with an intact DNA-PKcs encoding gene showed ASF1A phosphorylation following DNA damage (**Fig. 11K**). Given that ASF1A is phosphorylated by Tlks on serine 166, 175, 192 and 199 in the C-terminal tail (Klimovskaia et al., 2014a) we examined whether mutation of these serines to alanine could block the damage-induced ASF1A phosphorylation. Indeed, expression of HA-tagged ASF1A with a S192A mutation blocked its ability to be phosphorylated in response to DNA damage, while wild type ASF1A-HA was efficiently phosphorylated in response to DNA damage (**Fig. 11L**). As such, ASF1A is phosphorylated on serine 192 by DNA-PKcs in response to DSB induction.

**Figure 11**

**A**

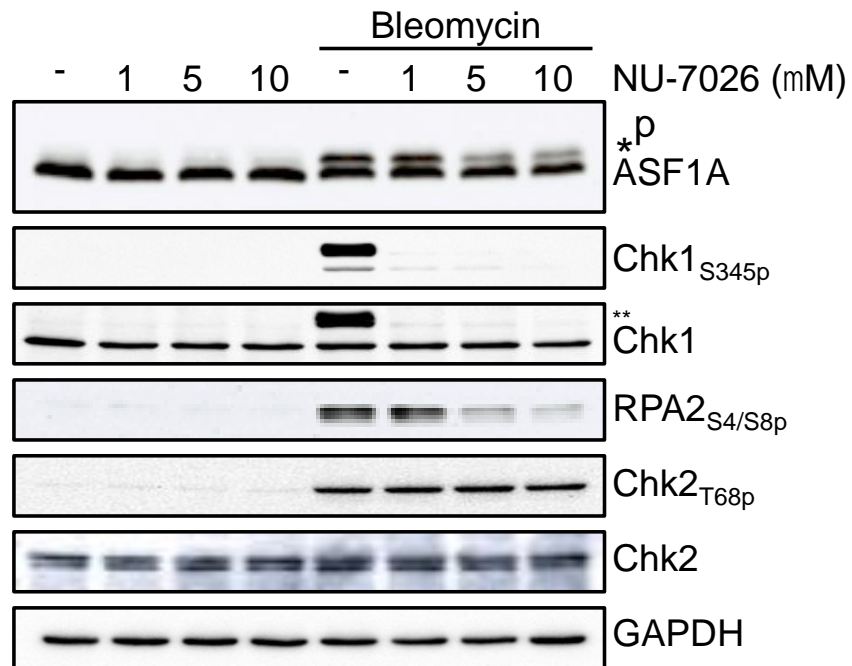




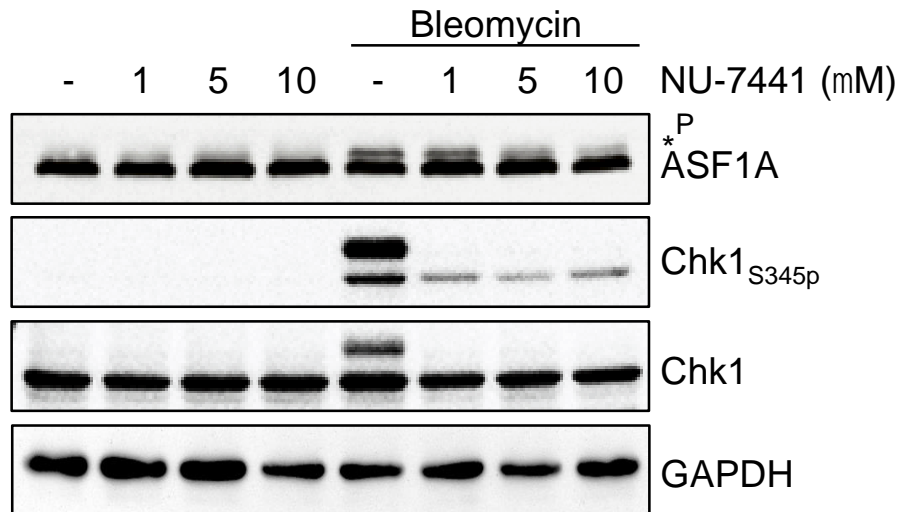




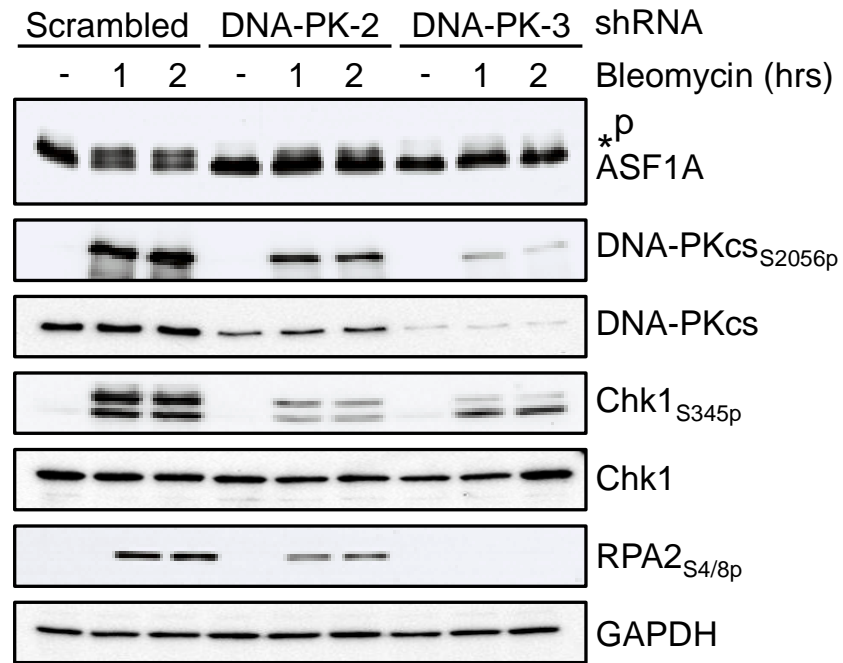
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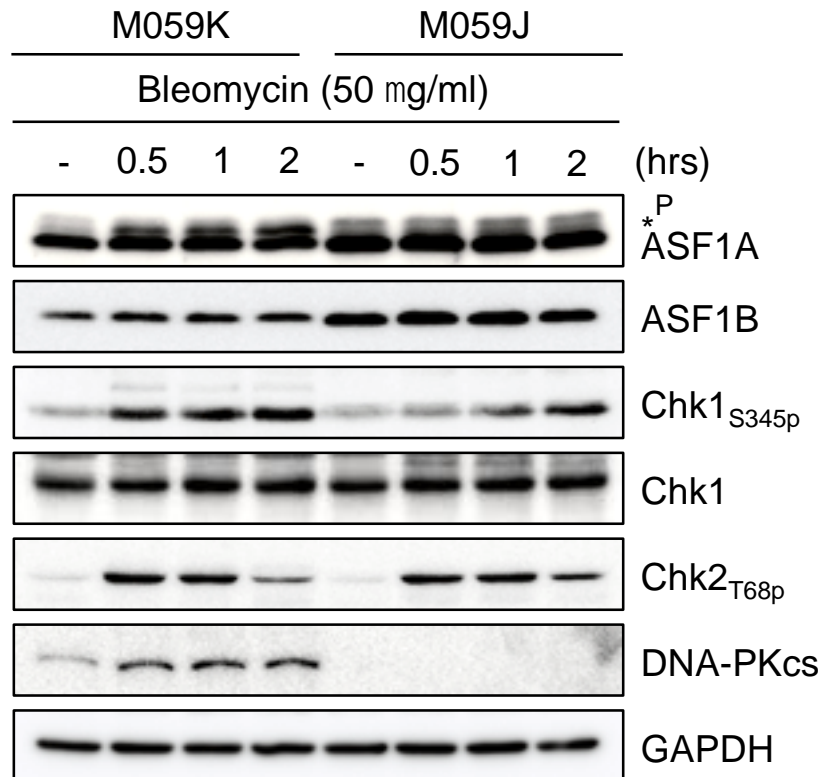
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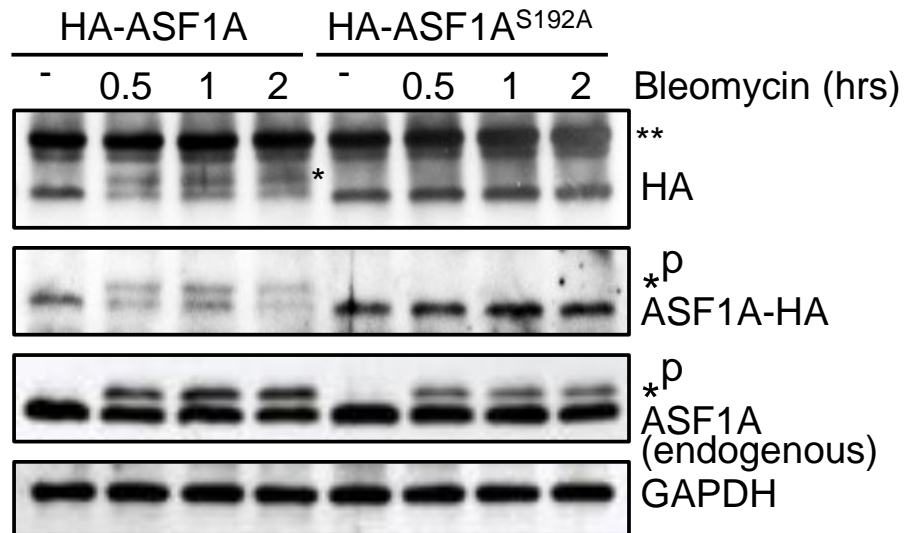
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**K**



L



**Figure 11. ASF1A is phosphorylated at serine 192 by DNA-PKcs following DSB damage. (A) Post-translational modification of ASF1A in response to DNA damage.** HeLa cells were treated with increasing concentrations of bleomycin for 15 and 30 minutes and analysed by Western blotting. Chk1 phosphorylation serves as a DNA damage marker. The asterisk indicates ASF1A phosphorylation. **(B) Induction of ASF1A phosphorylation by Doxorubicin.** An increased dose of Doxorubicin was applied to damage HeLa cells for 15 or 30 minutes. p53<sub>S15p</sub> served as an indication of DNA damage. **(C) ASF1A phosphorylation in MEF cells.** MEF cells were treated with bleomycin and NCS for 30 minutes. **(D) ASF1A is phosphorylated in response to DSB damage.** Lysates from cells treated with or without

bleomycin were subject to either alkaline phosphatase (NEB) alone or a combination of phosphatase and PhosSTOP phosphatase inhibitor cocktail (Roche) at room temperature for 10 minutes followed by Western blotting analysis. (E) Rapid inactivation of Rad9 during DNA damage. (F) DSB-induced ASF1A phosphorylation is not mediated by ATM. Cells were treated with 4  $\mu$ g/ml KU-55933 for one hour followed by co-treatment with bleomycin for an additional hour. (G) DSB-induced ASF1A phosphorylation is not mediated by ATR. The experiment was performed as in F, with the 4  $\mu$ g/ml ATR inhibitor VE-821. (H) DSB-induced ASF1A phosphorylation requires DNA-PK. Inhibition of DNA-PK by 3  $\mu$ g/ml NU-7026 as described in F. The two asterisks indicate that the antibody against phosphorylated Chk1 was not effectively stripped from the previous immunodetection. (I) The addition of DNAPK inhibitors resulted in the reduction of ASF1A phosphorylation. Following 1-hour pre-incubation with various doses of NU-7441, cells were challenged by bleomycin for additional one hour. Chk1<sup>S345p</sup> was probed in order to monitor DNA-PKcs activities. (J) Control and two stable DNA-PKcs knockdown cell lines were incubated with or without bleomycin and analysed by Western blotting. DNA-PKcs, Chk1 and RPA2 phosphorylation were used as DNA damage markers. (K) Comparison of ASF1A phosphorylation between parental (M059K) and DNA-PKcs deficient (M059J) cell lines to confirm the involvement of DNA-PKcs. Phospho Chk1 and Chk2 which are DNAPK and ATM downstream targets respectively, were blotted to rule out ATM in this pathway. (L) DSB-

induced ASF1A phosphorylation occurs on serine 192 (S192). Western blot analysis of ASF1A phosphorylation in cells transiently expressing either C-terminal HA-tagged ASF1A wild type or phospho-block S192A construct for 24 hours. The two asterisks indicate a non-specific band picked up by the HA antibody. 50  $\mu$ g/ml bleomycin was used in E-L.

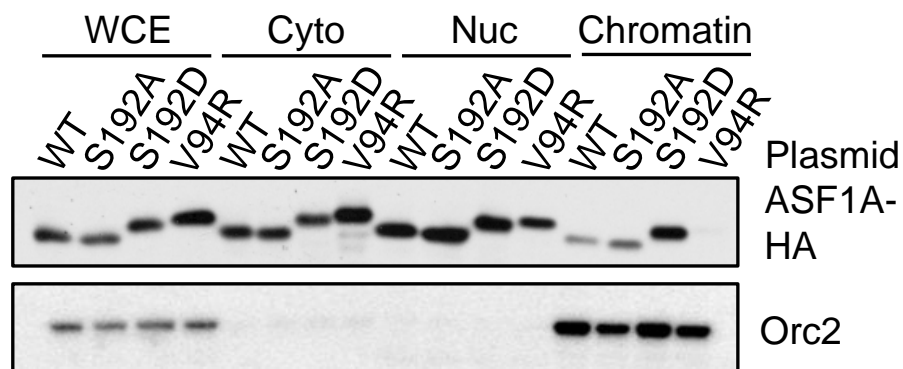
### 3.2.8. ASF1A phosphorylation facilitates its chromatin localization and histone binding affinity

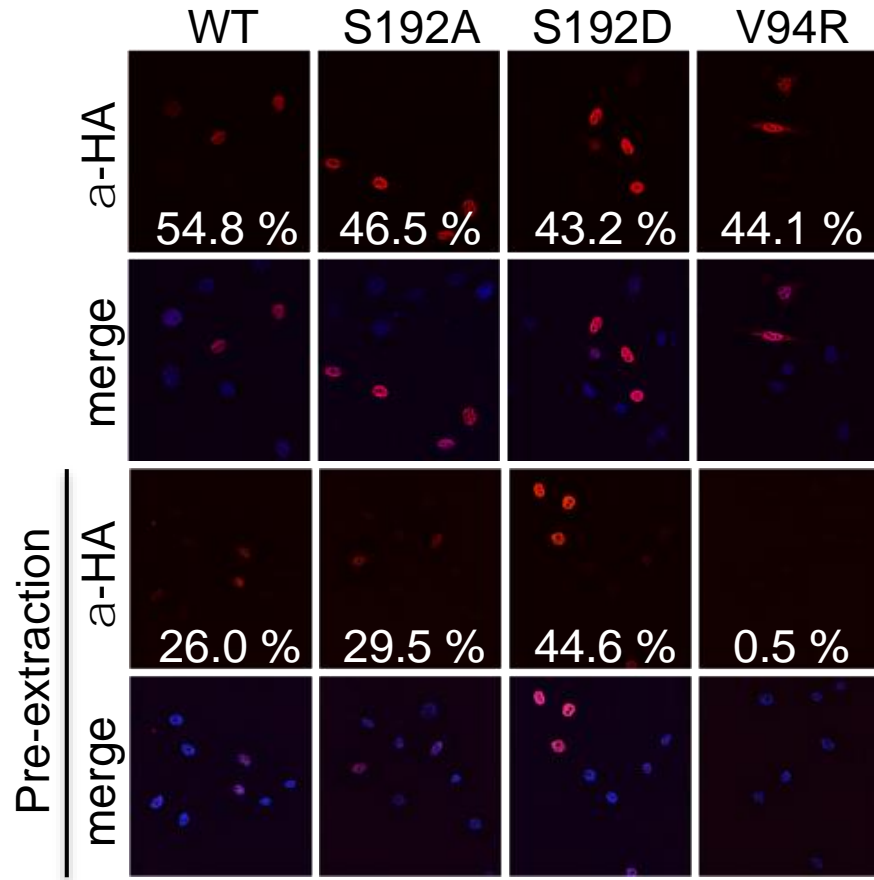
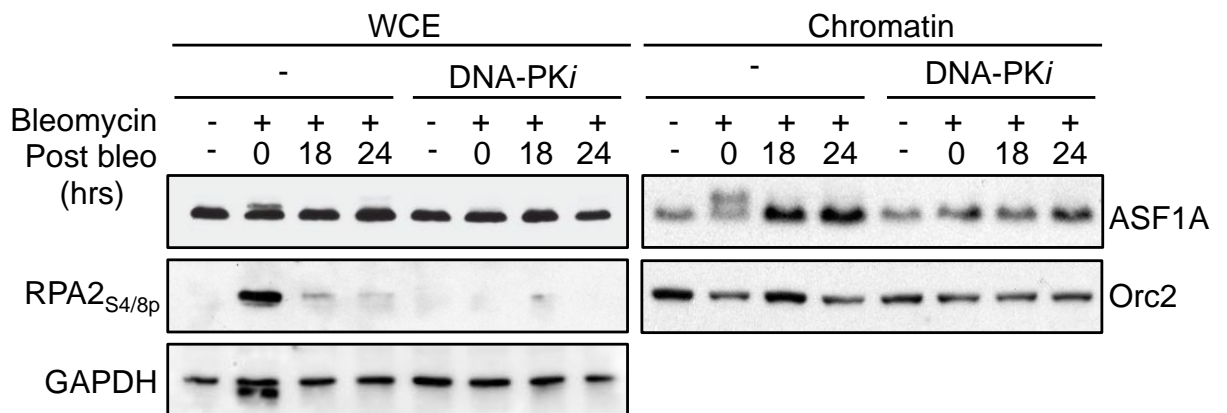
We examined the potential function of damage-induced ASF1A phosphorylation by mutating serine 192 to aspartic acid to mimic permanent phosphorylation. Chromatin fractionation of cellular extracts shows that ASF1 was recruited to chromatin, and this depended on its ability to bind to histones H3/H4 because the ASF1A<sup>V94R</sup> mutant which loses histone binding ability (Antczak et al., 2006) did not interact with chromatin (**Fig. 12A**). While comparable levels of ASF1A<sup>WT</sup> and ASF1A<sup>S192A</sup> were bound to chromatin, the ASF1A<sup>S192D</sup> showed greater binding to chromatin (**Fig. 12A**), suggesting that the damage induced ASF1A phosphorylation promotes ASF1A recruitment to chromatin. When cells were pre-extracted with Triton-X to extract soluble ASF1A followed by immunofluorescence analysis, 44.6% of the cells had significant ASF1A<sup>S192D</sup> retention on the chromatin, as compared to 26 or 29.5% for ASF1A<sup>WT</sup> and ASF1A<sup>S192A</sup> respectively (**Fig. 12B**). Given that the non-histone binding ASF1A<sup>V94R</sup> mutant was maintained on chromatin in only about 0.5% of cells, chromatin association of ASF1 is likely to reflect its role in chromatin assembly. As such, it appears that the phosphomimetic of ASF1 is likely to promote chromatin assembly. In agreement, we observed that phosphorylated ASF1 was selectively enriched on chromatin during induction of DSBs (**Fig. 12C**). Furthermore, elevated levels of ASF1 remained on the chromatin at 18 or 24 hours after bleomycin treatment, although it was no longer phosphorylated. This recruitment and retention of ASF1 on chromatin is likely to depend on prior

ASF1A S192 phosphorylation, because it was largely blocked upon DNA-PK inhibition (**Fig. 12C**). Consistent with ASF1A S192 phosphorylation promoting the replication-dependent chromatin assembly pathway, we also observed that ASF1A<sup>S192D</sup> had enhanced interactions with histone H3.1, H4, and CAF-1 by immunoprecipitation analysis, in comparison to ASF1A<sup>WT</sup> and ASF1A<sup>S192A</sup> (**Fig. 12D**). Furthermore, ASF1A<sup>S192D</sup> also promoted interaction with MMS22L (**Fig. 12D**). By contrast, ASF1A<sup>S192D</sup> did not increase interaction with H3.3, the replication-independent histone variant (**Fig. 12E**). Consistent with the idea that ASF1 mediated chromatin assembly and MMS22L interaction is upregulated after DSB induction in a manner dependent on ASF1A phosphorylation, we observed an increase in the amount of histones and MMS22L bound to wild type ASF1 after DSB induction, but this did not occur in the S192A mutant (**Fig. 12F**). Taken together, these results suggest that DNA damage-induced ASF1A phosphorylation may promote chromatin assembly and MMS22L interaction.

**Figure 12**

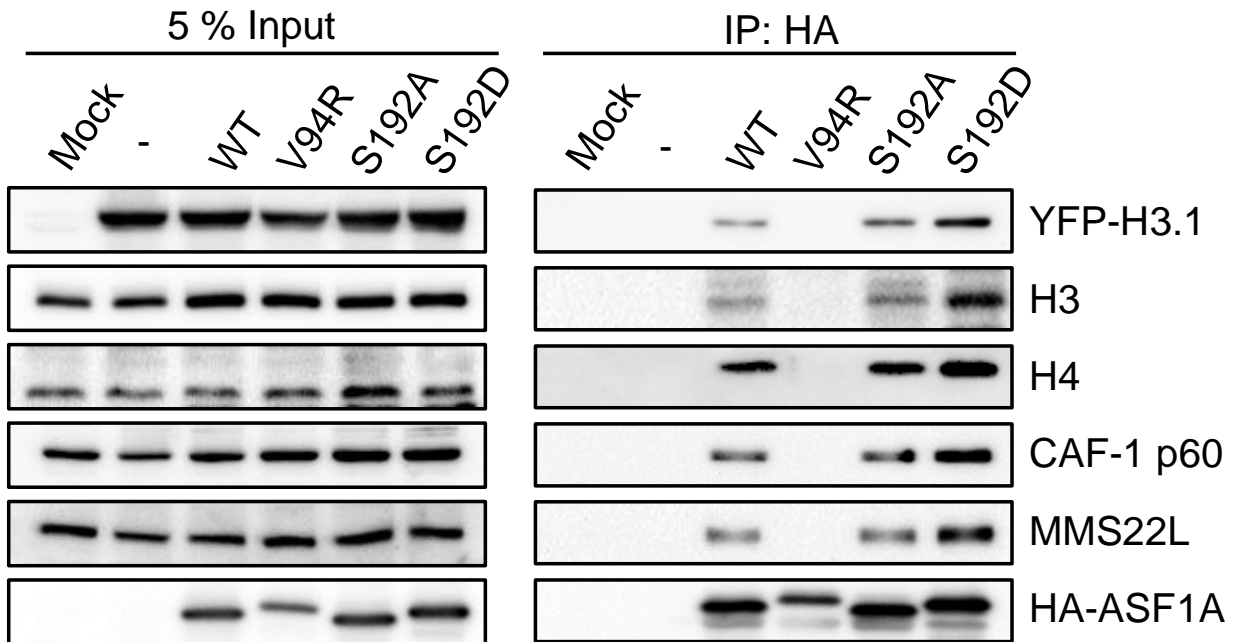
**A**



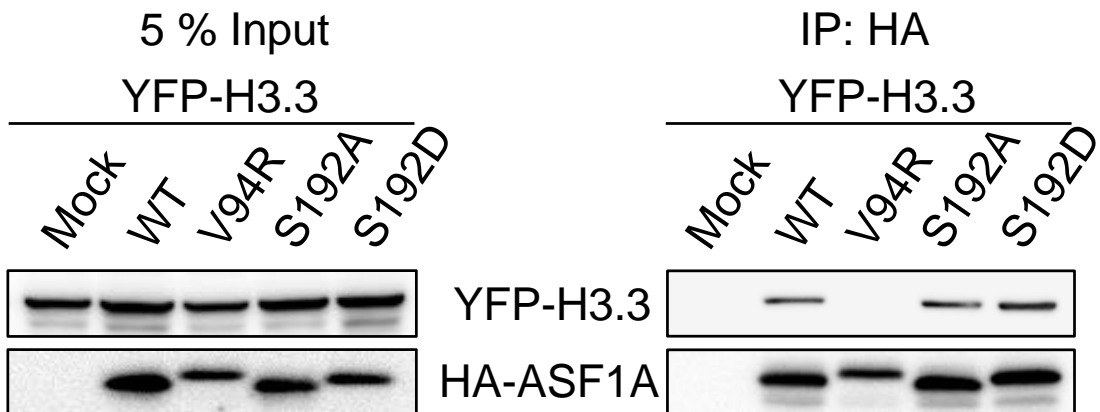
**B****C**



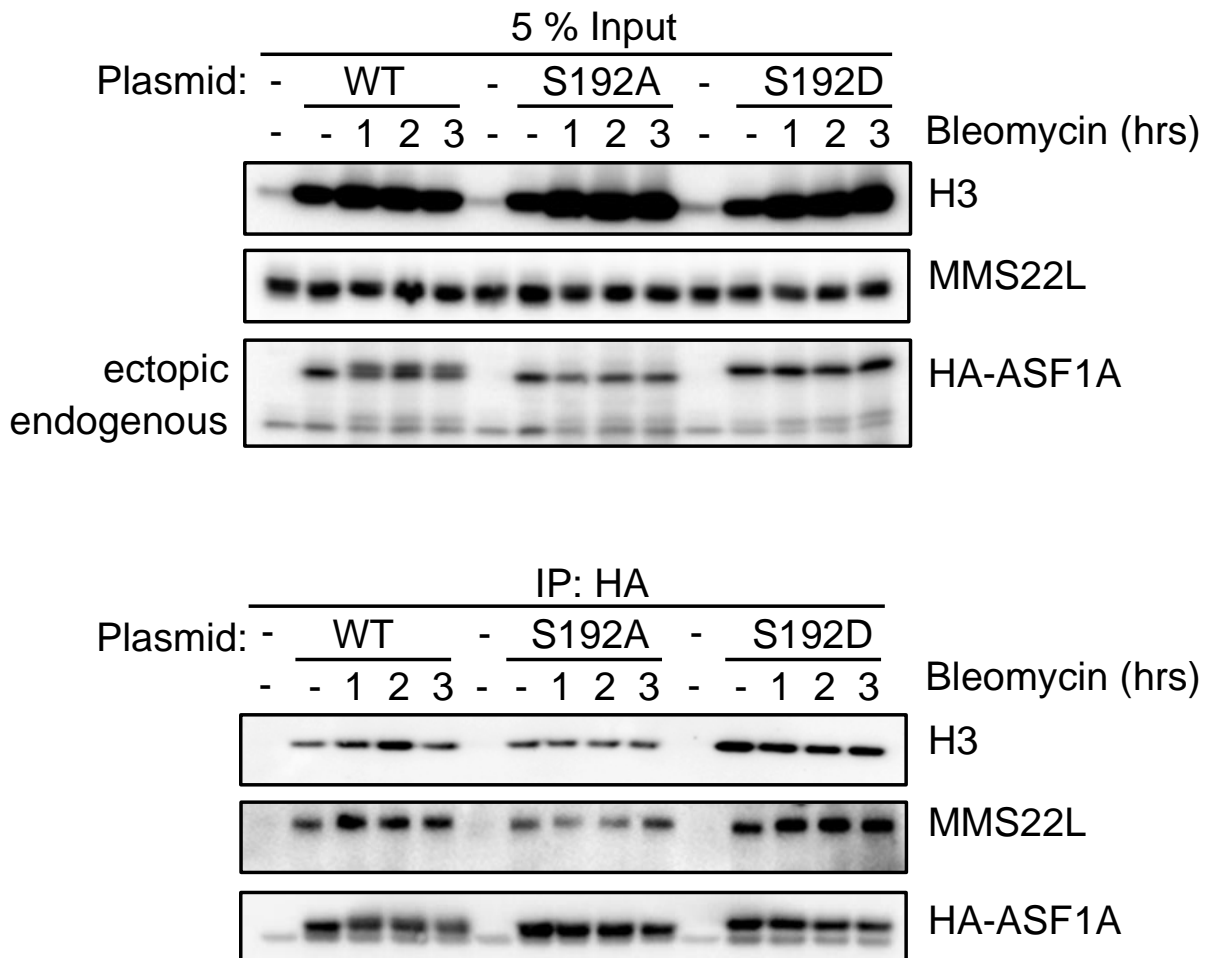
D



E



**F**



**Figure 12. Phosphorylation of ASF1A promotes its retention on chromatin and enhances its association with histones. (A)** ASF1A binding to chromatin is enhanced by phosphorylation. HA-tagged wild type and mutant ASF1A proteins were transiently expressed in HeLa cells for 24 hours. A portion of each sample was saved as whole cell extract (WCE) and

the rest was fractionated into cytoplasmic (Cyto), nuclear and chromatin fractions. Orc2 serves as a control for a known chromatin associated protein. (B) ASF1A binding to chromatin is enhanced by phosphorylation. Cells with transient wild type or mutant ASF1A expression were either subject to immunostaining analysis without pre-extraction to measure transfection efficiency or with pre-extracting with 0.1% Triton X-100 before fixation to remove soluble ASF1A protein, allowing the observation of the chromatin-bound form. 100 cells per sample were counted from two independent experiments to determine the percentage of cells with detectable levels of HA-ASF1. (C) Inhibition of DNA-PKcs prevents ASF1A recruitment to chromatin induced by DNA damage. Cells were pre-treated with 10  $\mu$ M DNA-PK inhibitors (NU-7026) for 30 minutes followed by an additional 2-hour co-incubation with 40  $\mu$ g/ml bleomycin. After drug removal, NU-7026 was re-applied for the indicated time periods. RPA2<sup>S4/8p</sup> served as an indication of DNA-PKcs activity. (D) Association of mutant ASF1A proteins with histones and other chromatin factors was examined by immunoprecipitation analysis. HeLa cells with wild type or mutant ASF1A expression were co-transfected with YFP tagged H3.1 for 24 hours. “Mock” refers to cells treated with lipofectamine 2000 transfection reagent alone. (E) Phospho-mimetic ASF1A mutant (ASF1A<sup>S192D</sup>) exhibits increased association with histone H3.3 interaction. Immunoprecipitation of HA-tagged ASF1A mutated proteins with ectopic histone H3.3. HA-tagged ASF1A plasmids were co-transfected with YFP-tagged H3.3 into HeLa cells

for 24 hours followed by the IP protocol described in material and methods.

(F) Impact of DNA damage on ASF1A interaction with H3, CAF-1 and MMS22L upon 20  $\mu\text{g/ml}$  Bleomycin treatment. Cellular lysates for HA immunoprecipitation were prepared as described in Materials and Methods. Samples with the double “-“ labelling are “untreated” cellular lysates.

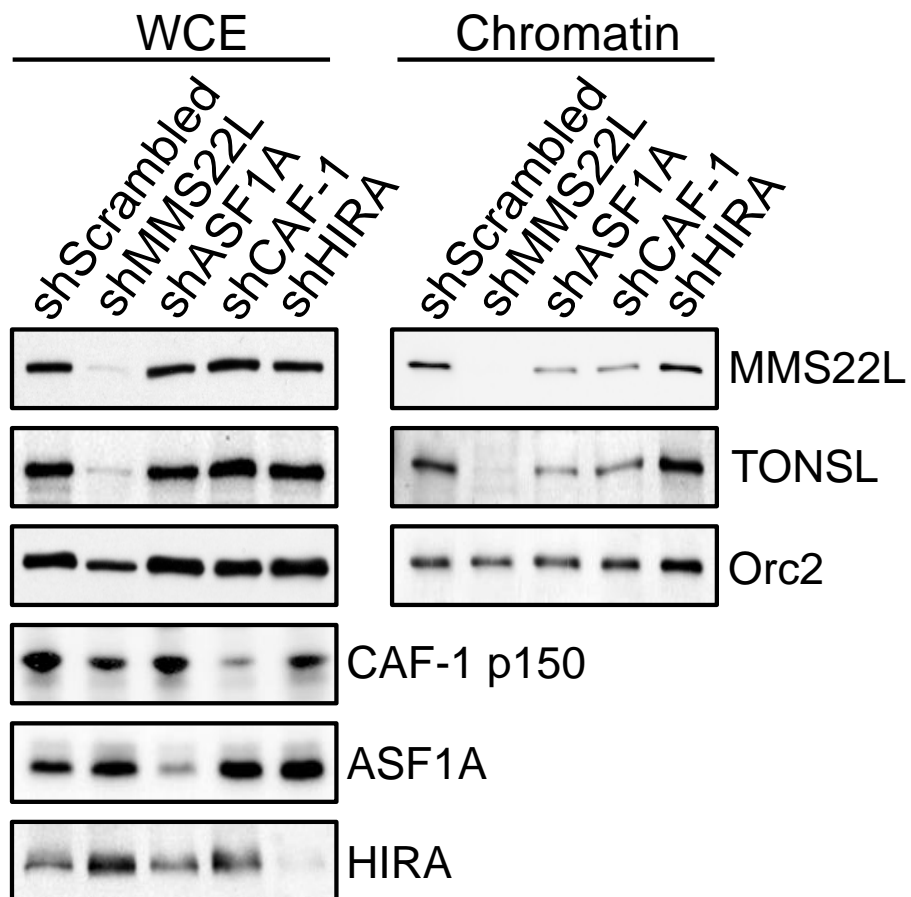
### **3.2.9. Damage induced ASF1 phosphorylation promotes MMS22L recruitment through newly-assembled histones to facilitate Rad51 loading**

Given that DSB-induced phosphorylation of ASF1A enhances ASF1A's interactions with chromatin and MMS22L (**Fig. 12**), we tested whether ASF1A promotes MMS22L/TONSL recruitment to chromatin. We found that knockdown of ASF1A greatly reduced the amount of MMS22L and TONSL on chromatin normally (**Fig. 13A**) and following induction of DSBs (**Fig. 13B**). Recruitment of MMS22L/TONSL to chromatin appeared to be enhanced by the replication-dependent chromatin assembly pathway in general, because knockdown of CAF-1, but not HIRA, also reduced MMS22L/TONSL levels on chromatin (**Fig. 13A**). To further investigate whether chromatin assembly was required for MMS22L/TONSL-mediated recruitment of Rad51 to sites of DSB repair, we examined Rad51 recruitment in the ASF1A<sup>V94R</sup> mutant that is unable to assemble chromatin. Rad51 recruitment was greatly reduced in the ASF1A<sup>V94R</sup> mutant (**Fig. 13C**) indicating that histone binding and presumably chromatin assembly by ASF1A is required for Rad51 recruitment during HR. Furthermore, the damage-induced phosphorylation of ASF1A appears to also play a key role in HR because the ASF1A<sup>S192A</sup> mutant was as defective at Rad51 recruitment as the ASF1A<sup>V94R</sup> mutant that fails to bind histones. It has been shown previously that MMS22L/TONSL is recruited to ssDNA (O'Donnell et al., 2010). Our observation that DNA damage-induced ASF1A phosphorylation promotes chromatin assembly in order to recruit MMS22L/TONSL to achieve Rad51 loading onto ssDNA, suggests an unexpected model whereby chromatin is assembled

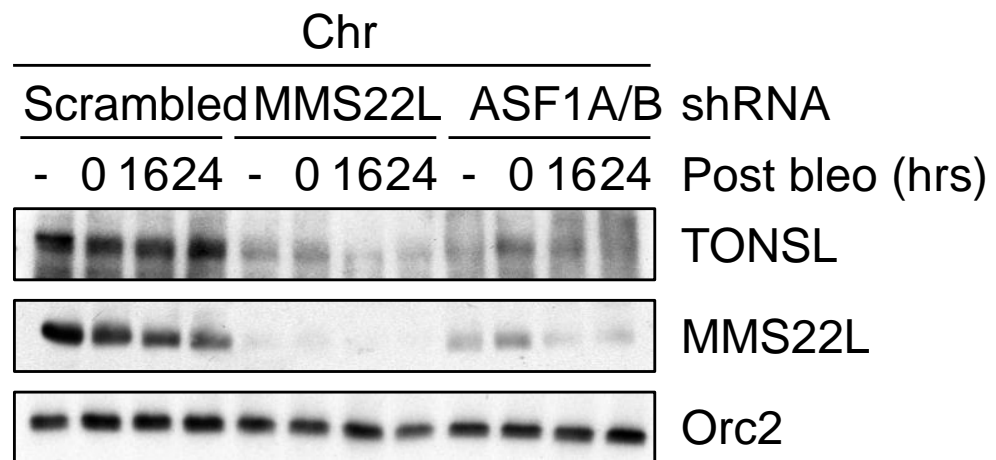
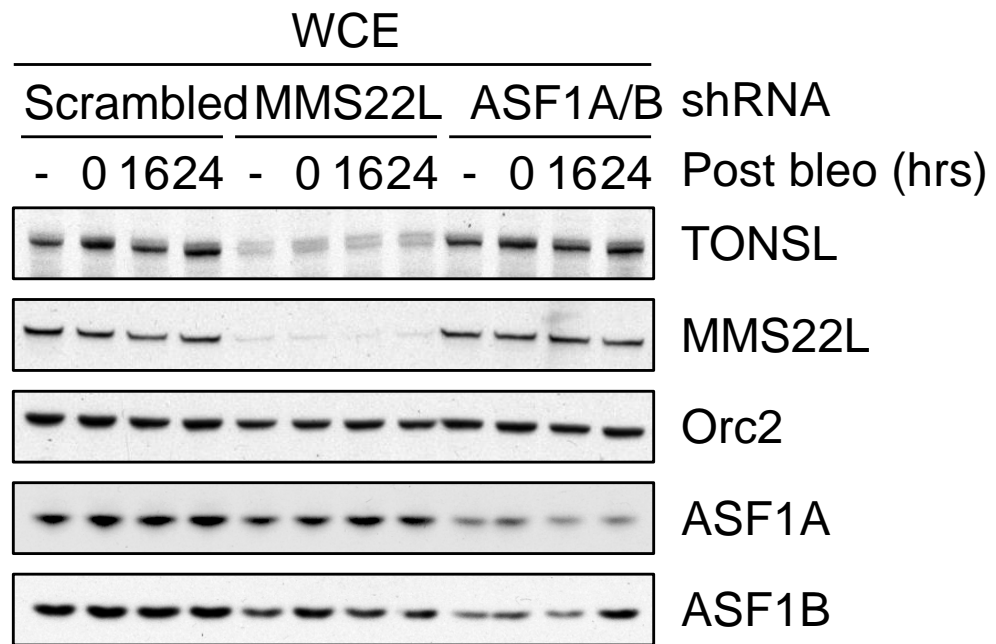
transiently in the midst of homologous recombination, potentially onto ssDNA, to promote DNA repair *per se* (**Fig. 13D**).

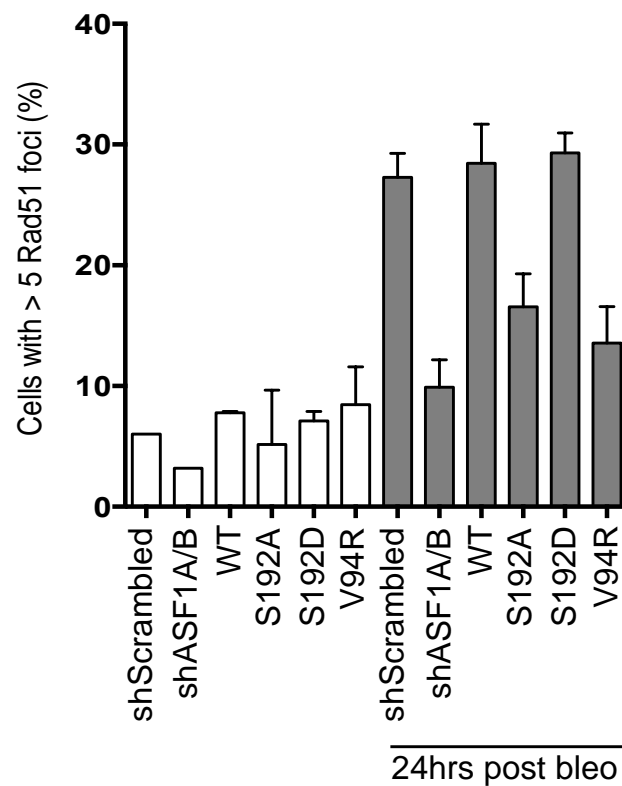
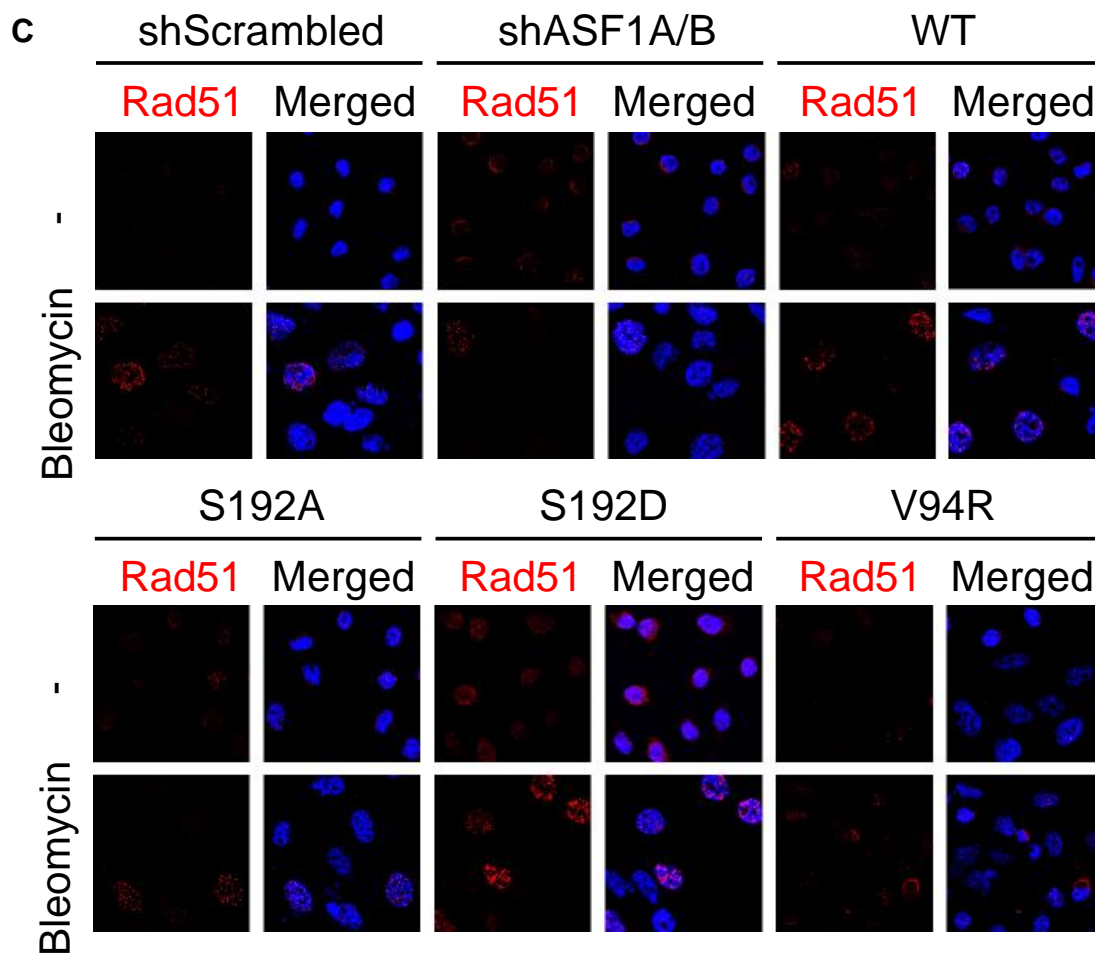
**Figure 13**

**A**



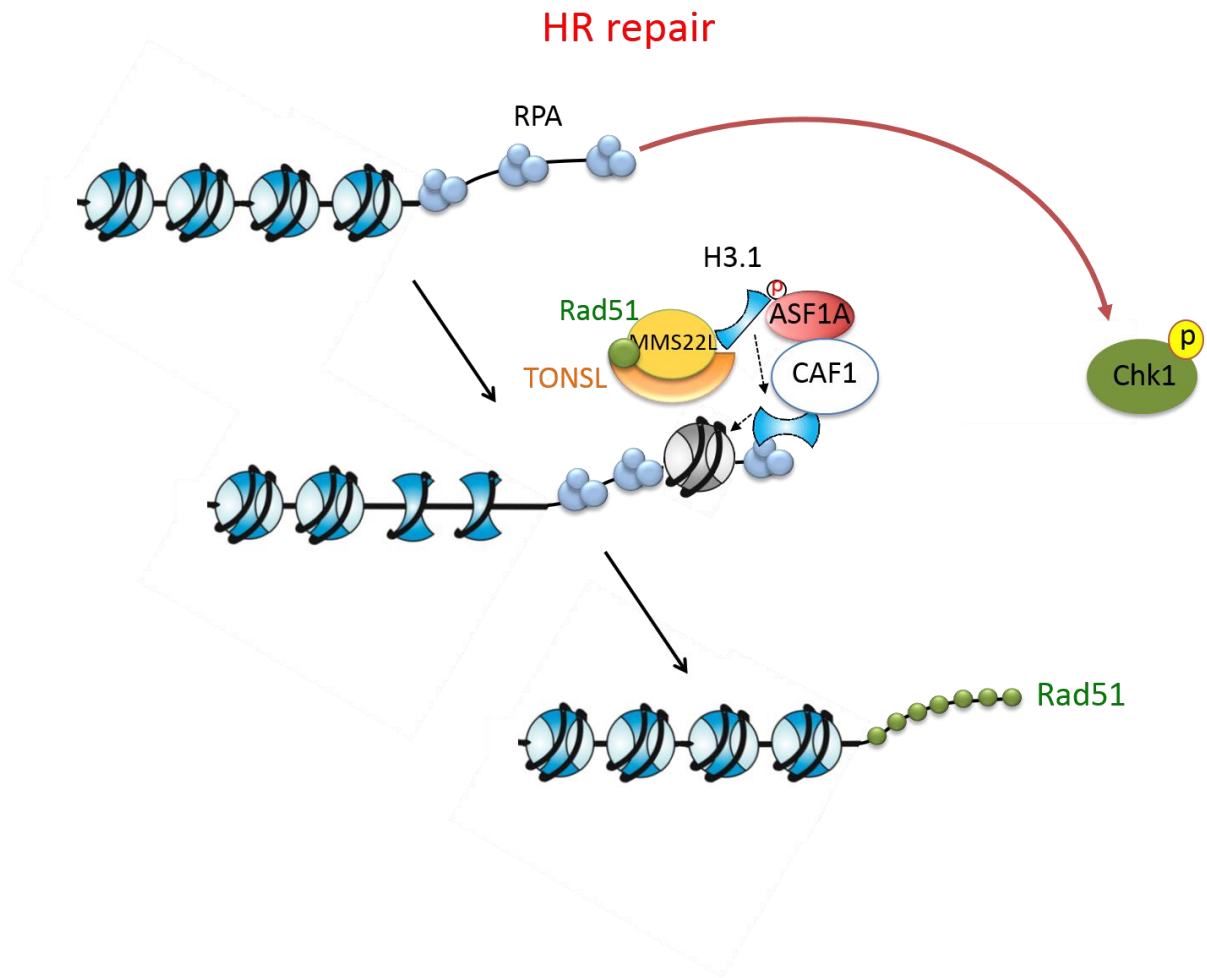
**B**







D



**Figure 13. ASF1A-mediated histone deposition is required for the recruitment of TONSL-MMS22L complex and Rad51 nucleofilament formation. (A) Level of TONSL-MMS22L complex in chromatin upon histone chaperone knockdowns, as described in Fig. 9A. (B) ASF1 also facilitates the recruitment of MMS22L/ TONSL during DNA damage. shScrambled, shMMS22L and shASF1A/B cells were harvested at the indicated recovering time points after 2-hour bleomycin (40  $\mu$ g/ml) treatment.**

Samples from the same batch were then prepared for whole cell extracts (WCE) and chromatin fractions (Chr). (C) Analysis of the ability of wild type and mutant ASF1A to restore Rad51 nucleofilament formation in ASF1 depleted cells during DNA repair. ASF1A/B depleted cells were transfected with shRNA-resistant HA tagged wild type and mutant ASF1A plasmids. 24 hours after transfection, cells were transferred to poly-L lysine coated coverslips and incubated overnight followed by addition of 40  $\mu$ g/ml bleomycin for 2 hours. Immunostaining was performed 24 hours after removal of bleomycin. Over 100 cells per sample per experiment were analysed for the number of Rad51 foci in the nucleus. Quantitation results were gained from three independent experiments and the average and standard deviation are shown. (D) Schematic model of histone loading-induced Rad51 nucleofilament formation. During DNA damage, DNA-PKcs-mediated ASF1A phosphorylation enhances nucleosome deposition through CAF-1 and helps with the recruitment of TONSL-MMS22L complex towards resected ssDNA. This action triggers Rad51 loading to replace RPA to enable strand invasion during homologous recombinational repair.

### **3.3. Discussion**

In this study, I have unveiled an unexpected intrinsic role for replication-dependent chromatin assembly in the fundamental mechanism of homologous recombinational repair. This chromatin assembly is likely to occur on the ssDNA that results from end resection of DSBs, where the newly-synthesized,

unmethylated histones deposited onto the DNA function to recruit MMS22L/TONSL to replace RPA with Rad51, to form the nucleofilament that mediates strand invasion. Furthermore, this function is promoted by ASF1A phosphorylation in response to DSBs. Our results suggest that dynamic chromatin assembly plays an active role in the DNA repair process *per se*.

Consistent with MMS22L/TONSL functioning in the same pathway as replication-dependent chromatin assembly to achieve Rad51 loading onto DNA, ASF1 and CAF-1 depletion phenocopied MMS22L knockdown for defects in the DDR. The extent of the HR defect observed for ASF1 knockdown (**Fig. 8G**) was very similar to that reported upon MMS22L and TONSL knockdown using the ISceI HR reporter system (O'Donnell et al., 2010). Indeed, the DNA damage repair defects for Rad51, MMS22L, TONSL, CAF-1 and ASF1 knockdown were indistinguishable by comet assay (**Fig. 10E**). In agreement, MMS22L, ASF1 and CAF-1 knockdown all led to the same degree of persistent activation of the ATR-RPA-Chk1 pathway and cell cycle arrest (**Figs. 3, 4, 7, 9**). MMS22L, TONSL, ASF1, or CAF-1 knockdown, or an ASF1 mutant that cannot be phosphorylated by DNA-PKcs or an ASF1 mutant that cannot bind histones all resulted in a similar degree of defect in Rad51 loading (**Figs. 10, 11D, 12C**). Furthermore, ASF1 and CAF-1 function upstream of MMS22L/TONSL in the Rad51 loading pathway, because ASF1 and CAF-1 are required for recruitment of MMS22L/TONSL to chromatin (**Fig. 13A**). Taken together, all these data indicate that replication-dependent chromatin assembly is required for MMS22L/TONSL mediated Rad51 loading onto ssDNA during HR.

### **3.3.1. Rad51 loading is promoted by chromatin assembly onto ssDNA**

I propose that assembly of chromatin occurs onto ssDNA during HR for the following reasons. The evidence that MMS22L/TONSL is recruited to RPA-bound ssDNA produced from DSB processing is compelling (Piwko et al., 2016), as is the evidence that MMS22L/TONSL is recruited to chromatin via an interaction with recently deposited histones (Saredi et al., 2016). My work synthesizes these observations to suggest that chromatin is assembled transiently onto RPA-bound ssDNA to recruit MMS22L/TONSL to facilitate homologous recombination. Previous analyses from the Durocher laboratory (O'Donnell et al., 2010) observed a near perfect accumulation of MMS22L and TONSL at sites of DNA damage marked by RPA, contained within  $\gamma$ H2AX-marked domains. These RPA containing subchromatin microcompartments have been shown previously to be delineated by ssDNA (Bekker-Jensen et al., 2006), indicating that MMS22L/TONSL colocalize with ssDNA formed following DSB processing. Indeed, depletion of CtIP, a protein that plays a key role in end resection (Sartori et al., 2007), profoundly reduced MMS22L/TONSL recruitment to sites of DNA damage (O'Donnell et al., 2010). Furthermore, a recent study showed that MMS22L, which binds directly to Rad51, is recruited to ssDNA by RPA (Piwko et al., 2016). Given that TONSL serves as a reader of H4K20me0 (Saredi et al., 2016), which occurs only transiently on newly-synthesized histones after their assembly onto DNA by ASF1 and CAF-1, my observed requirement for

ASF1 and CAF-1 for recruitment of MMS22L/TONSL to ssDNA is consistent with chromatin assembly onto ssDNA following DNA resection.

Several previous studies support the ability of nucleosomes to assemble onto ssDNA. Nearly 40 years ago, Bruce Alberts reconstituted nucleosomes onto ssDNA that resembled nucleosomes on dsDNA by electron microscopy (Palter et al., 1979). Chromatin immunoprecipitation analysis at an unrepairable HO endonuclease site showed that yeast deleted for the *ARP8* gene, encoding a subunit of the INO80 ATP-dependent chromatin remodeler, maintain histone occupancy around the DNA lesion even though DNA resection and RPA recruitment apparently occurred normally, suggesting a possible transient association between nucleosome and ssDNA *in vivo* (Tsukuda et al., 2005). Most recently, DNA resection was shown to be able to occur *in vitro* on nucleosomal templates, and that histones remain associated with the ssDNA after DNA resection (Adkins et al., 2017). I propose that nucleosomes assembled onto resected ssDNA serves as a platform to specifically recruit DNA repair machineries, such as MMS22L/TONSL.

### **3.3.2. Extensive DSB end resection in the absence of ASF1**

Unexpectedly, I observed extensive end processing of DSB into ssDNA in ASF1 depleted cells, equivalent to the levels in cells depleted of 53BP1 (**Fig. 8E**). The resection that I observed is strictly dependent on induction of DSBs and is therefore distinct from the increase in ssDNA that has been reported previously in the absence of ASF1 (Groth et al., 2007). There are several possible reasons

for the extensive DNA end resection that occurs after DSB damage induction in ASF1 depleted cells. Firstly, it is possible that end-resection continues when the Rad51 loading step and subsequent strand invasion is blocked. To determine this possibility, future experiments should measure whether end resection increases upon Rad51 knockdown. Alternatively, the increased amount of DNA resection in the absence of ASF1 could be because the chromatin template in general has a lower nucleosome density (O'Sullivan et al., 2014). If so, this would indicate that the normal nucleosomal density within our cells serves to restrain DNA resection.

### **3.3.3. ASF1/CAF-1 promote ATM activation possibly through histone H4 acetylation on lysine 16**

In contrast to the ATR-Chk1 pathway, which is persistently activated in DNA damage-stimulated ASF1 and CAF-1 knockdown cells, the ATM-Chk2 pathway is unexpectedly less sensitive to DNA damaging agents. How ASF1/CAF-1-mediated chromatin assembly affects ATM recruitment and activity is unresolved in this thesis. One explanation could be the failure of restoring the level of H4K16ac in ASF1 and CAF-1 depleted cells during DNA repair. Acetylation of H4 on lysine 16 can be mediated directly by the acetyltransferases hMOF, Tip60 and indirectly affected via chromatin remodelers Chfr and RNF8 as well as DMAP1 which is a member of Tip60 complex (Penicud and Behrens, 2014; Sharma et al., 2010; Sun et al., 2005; Wu et al., 2011). Intriguingly, these proteins have been reported to play roles in the activation of ATM to some degree. However, like,

Chfr and RNF8, ASF1 and CAF-1 do not have acetyltransferase activity but still affect the level of H4K16ac, indicating that the consequence of reduced H4K16ac in both ASF1 and CAF-1 depleted cells may result from a subsequent effect of altered nucleosome arrays or other possible histone modifications. It has been shown that downregulation of H4K16ac modulates chromatin relaxation (Rea et al., 2007) which may restrict ATM access to damaged DNA. What mechanism gives rise to the H4K16ac reduction in cells lacking ASF1 and CAF-1 is unclear. Both ASF1 and CAF-1 facilitate the restoration of H4K16ac during DNA repair whereas only ASF1 affects preexisting levels of H4K16ac. Asf1 has been shown to physically interact with the SAS complex that is the yeast homolog of human Tip60 (Osada et al., 2001), suggesting a possibility that ASF1 cooperates with the H4 acetyltransferase before depositing newly-synthesized histones onto chromatin via CAF-1.

#### **3.3.4. DNA-PK mediated phosphorylation of ASF1 drives chromatin assembly during DSB repair**

It may be unexpected but not surprising that DNA-PK serves as a kinase for ASF1A phosphorylation. DNA-PK binds to and is activated by DNA ends and is abundant in human cells (Meek et al., 2008), making it an ideal kinase to stimulate a phosphorylation event specifically in response to DSBs. It seems logical that in most cases DNA-PK will be the first repair factor to bind a DSB. Just because DNA-PK is a NHEJ factor, that doesn't automatically confound its use in promoting chromatin assembly during HR. There are clear examples of co-localization of NHEJ and HR factors at the same DNA lesion (Schwartz et al.,

2005). The fact that DNA-PKcs phosphorylates ATM, which is recruited by the HR factors Mre11-Rad50-Nbs1, indicates that both HR and NHEJ factors bind to the same DNA ends (Zhou et al., 2017). DNA-PK mediated ASF1A<sup>S192</sup> phosphorylation alone stimulates its accumulation on chromatin and is sufficient to augment formation of complexes with histones, CAF-1, HIRA and TONSL-MMS22L (**Fig 6D**). Future studies will determine exactly how phosphorylation of ASF1 on S192 in the unstructured C-terminus of ASF1 promotes these interactions.

It is possible that DNA-PK is not the only kinase for ASF1A phosphorylation in response to damage, given that this phosphorylation promotes efficient Rad51 loading while DNA-PK is not required for HR. For example, it is known that the DNA-PK deficient M059J cell line has reduced ATM activity. In addition, we find that knockdown of DNA-PK can also affect ATM activity. It is possible therefore that ATM or some of its downstream kinases may also phosphorylate ASF1 in a redundant manner with DNA-PK. For that matter, it is of interest that the sequence around ASF1A S192 fits the consensus site for Chk2.

### **3.3.5. ASF1 function may prevent DNA-PKcs from sliding along DNA during the process of NHEJ repair**

Besides ATR and ATM being affected when we impeded chromatin assembly, DNA-PKcs status was perturbed as well. However, I didn't observe a significant increase or defect in NHEJ repair efficiency despite the robust



accumulation of DNA-PKcs on chromatin in ASF1-depleted cells (**Fig. 11**). Ku physically serves as a DNA binding subunit for DNA-PKcs and it is known that instead of binding to DNA termini, the Ku heterodimer has the ability to slide along the duplex DNA to internal positions (de Vries et al., 1989; Yaneva et al., 1997). In addition, Ku also associates with nucleosomes but this doesn't seem to affect DNA-PKcs kinase activity (Park et al., 2003). However, Ku doesn't possess the energy to overcome the nucleosomal barrier, leading to its binding being limited to DNA ends (remaining within the 20~30bp distal to a break site) (Park et al., 2003). In agreement with this observation, DNA-PK and XRCC4 were greatly enriched on DSB ends around which nucleosome density is presumably lower in cells lacking ASF1 (**Fig. 9**). Therefore, I propose that timely nucleosome deposition executed by ASF1 / CAF-1 during the process of NHEJ provides a spatial barrier to position NHEJ complex at the tip of DNA breaks.

### **3.3.6. Chromatin assembly on ssDNA mediated by ASF1/CAF-1 promotes homologous recombinational repair**

In agreement with chromatin assembly occurring in the midst of DSB repair, several studies demonstrate the need for chromatin compaction at break sites following chromatin expansion/ disassembly to effectively facilitate DNA repair (Kruhlak et al., 2006; Smeenk and van Attikum, 2013). In line with this notion, p150 CAF-1 has been shown to promote laser-induced Rad51 nucleofilament formation by transiently recruiting HP1 (Baldeyron et al., 2011) which preferentially binds H3K9me3; meanwhile, depletion of CAF-1 in different species

also compromises HR repair (Lewis et al., 2005; Song et al., 2007). We propose that local assembly of unmodified newly-synthesized histones facilitates the recruitment of specific repair factors, such as MMS22L/TONSL at exactly the time that they are needed to bring Rad51 onto the ssDNA coated with RPA. It may be relevant that FACT, which is an H2A-H2B chaperone, associates with RPA (VanDemark et al., 2006), and it will be interesting to investigate if FACT is also required for efficient Rad51 loading and so could potentially be involved in the chromatin assembly onto ssDNA. It is possible that the RPA itself plays a stimulatory role in driving chromatin assembly onto the RPA-ssDNA, given that the RPA complex binds H3 to facilitate nucleosome assembly in biochemical experiments (Liu et al., 2017). In this biochemical study, the chromatin assembly occurred onto naked dsDNA adjacent to the RPA-ssDNA, but the assays included insufficiently long ssDNA to form a nucleosome on ssDNA.

It is likely that the histones that are assembled by CAF-1 and ASF1 onto ssDNA during HR are removed upon Rad51 binding. In agreement, the ATP-dependent nucleosome remodeler p400 and Rad51 are both required for histone removal from around an induced AsiSI site in human cells (Courilleau et al., 2012). While RPA loading and DNA resection occur normally in the absence of p400, Rad51 is not recruited and histones are not removed from around the DSB (Courilleau et al., 2012). These data suggest that p400 disrupts the nucleosomes from ssDNA to enable Rad51 loading onto the ssDNA. The ATP-dependent nucleosome remodeler BRG1 also promotes removal of RPA and replacement with Rad51 (Qi et al., 2015), so could also be involved in either the chromatin

assembly onto ssDNA or chromatin disassembly from the ssDNA. We propose a model where chromatin is disassembled around a DSB to enable DNA resection and RPA loading. Next, chromatin is transiently reassembled onto the ssDNA to recruit Rad51, which itself drives the displacement the histones. After strand invasion and DNA synthesis have occurred, histones are reassembled once again onto dsDNA to complete HR (**Fig. 13D**).

In summary, genomic processes such as DSB repair are facilitated by the local removal of histones from the DNA, to provide access to the DNA lesion. However, an unanticipated aspect of the maintenance of genomic stability revealed by this study is the need for dynamic chromatin reassembly in the midst of homologous recombination, seemingly onto ssDNA, to promote the specific recruitment of the MMS22L/TONSL repair machinery in order to recruit Rad51 to ssDNA. These histones are likely themselves rapidly displaced during Rad51 incorporation onto the ssDNA during Rad51 nucleofilament formation. Such an active and dynamic role for chromatin assembly to drive the intrinsic steps of DNA repair is unprecedented.

## **Chapter 4**

### **Conclusions and future directions**

There is now a great deal of accumulated evidence showing that chromatin dynamics mediated by histone remodelers, histone chaperones as well as histone modifiers is pivotal to various aspects of genomic processes. Changes on chromatin or more specifically, the pattern of histone modifications and positioning of nucleosomes, provide not only epigenetic information on histones *per se* but spatial organization of the local architecture for cells to sense genomic alterations, for example, DNA damage. Even though the concept of the reciprocal association between chromatin and initiation of DNA repair pathways is not novel, whether nucleosome deposition is needed during the process of DNA repair is still unclear. In light of this concern, several studies have raised the concept of “expansion and condensation” of chromatin conformation during the DNA repair process; however, it is undiscerned whether nucleosome eviction and deposition occur sequentially or simultaneously around DNA lesions. In agreement with numerous findings, the work presented here demonstrated changes in chromatin dynamics (in this case by histone chaperones) affect the activation and recruitment of PIKK kinases. I discussed some potential mechanisms that may affect active status of ATM, ATR and DNA-PKcs from the perspective of lacking chromatin assembly in Chapter 3. The drawback of DNA damaging agents in experimental schemes is that they generate a variety of DNA lesions initiating even more complicated cellular responses. Therefore, it is inevitable in the future that studies will utilize site-specific DNA cutting and repair systems solely driven by HR (Zhou et al., 2014) and NHEJ pathways respectively for detail validations of how histone density or PTMs occurring in the absence of

histone chaperones contributes to the initiation / recovery of the DDR. For example, whether the level of H4K16ac around a DSB is required for the recruitment / maintenance of ATM at later stages of DDR and whether chromatin assembly during DNA damage induction is a constraint on DNA end processing and if this is the case, it is quantifiable to measure the length of DNA resection and determine new components regulating end resection in chromatin-dependent manner (Zhou et al., 2014).

I coincidentally found that DNA-PK serves as a kinase for ASF1A phosphorylation at serine 192 residue which is followed by a leucine (SL site). This DNA-PK dependent non-canonical SQ/TQ site has also been found in XLF, Ku80 and XRCC4 as well as DNA-PK itself (Lees-Miller and Meek, 2003; Yu et al., 2008). However, by the addition of selective small molecular inhibitors of ATM, ATR, Chk1 and DNA-PK (**Fig. 11F-I**), it is commonly questioned how specific the targeting drug is. Regarding there being a concern on the drug specificity, we speculate other kinases may function directly to phosphorylate ASF1A in a DNA-PK independent manner. Applying the siRNA knockdown or CAS9 conditional knockout systems to specifically eliminate the expression of PIKK or other candidates such as Chk1 and Chk2, one will be able to solidify this finding in the future.

The roles of ASF1A phosphorylation have been identified during DNA replication (Klimovskaia et al., 2014b) and in the progression of HR described in **Section 3.2.8-9**. With the inhibition of DNA-PK kinase activity, ASF1A failed to get phosphorylated and to reduce ASF1A chromatin recruitment after DNA

damage (**Fig. 12C**), illuminating a perspective of ASF1A phosphorylation that while encountering genomic attacks, cells are urgent to resolve the need for DNA damage-coupled chromatin assembly. Given that chromatin disassembly and assembly take place in the NHEJ pathway (Li and Tyler, 2016), our expanding interest from this point is to understand whether ASF1A phosphorylation participates in promoting NHEJ-related chromatin assembly by which one can test out the hypothesis proposed in **Section 3.3.5**. How the end joining repair machinery is displaced from repaired DNA has been a long-existing question in this field. Studies have shown histone deacetylation mobilizes NHEJ components and promotes repair (Miller et al., 2010; Roos and Krumm, 2016). Therefore, with the outcome of considerable enrichment of DNA-PKcs and XRCC around a DSB (**Fig. 9G**) caused by loss of a histone chaperone, it will be important to know whether the act of histone deposition or histone PTMs are responsible for dissipating the DNA-PK complex from intact chromatin after repair is complete.

In the absence of timely nucleosome occupancy at resected DNA, cells poorly performed the RPA to Rad51 transition nor efficiently recruited the HR repair machineries which further leads us to propose that nucleosome assembly on ssDNA participates in the progression of HR. A growing number of evidence (**Section 3.3.1**) supports the existence of histone-decorated ssDNA. However, the current knowledge of nucleosomal ssDNA only relies on *in vitro* observations whereas nucleosomes wrapped up into ssDNA *in vivo* may not yet be appreciated due to lack of proper techniques for its detection. We combined characteristics of TONSL, which recognizes newly histone H4 and promotes

Rad51 nucleofilament, with ASF1 that is a part of TONSL complex and presents similar phenotypes of stimulating HR repair to suggest the *in vivo* existence of nucleosomal ssDNA. We suggest that histone incorporation can occur to serve as a specific signal for recruiting DNA repair proteins or temporarily stabilize the unwound DNA structure. To directly prove histones exist on resected DNA, a system to isolate ssDNA by native BrdU IP from human cells needs to be established followed by probing for histones. In the future, we will continue to focus on understanding chromatin dynamics at ssDNA.

Collectively, this study has uncovered the unexpected importance of ASF1/CAF-1-mediated chromatin assembly in modulating activation of PIKK kinases and furthermore, suggests nucleosome deposition on ssDNA is a prerequisite to recruit the Rad51 loading machinery during a key intermediate step of HR repair.



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## **Vita**

Richard Ting-Hsiang Huang was born in Tainan, Taiwan, the second son of Min-Yao Huang and Shu-Ling Hu. After earning a Bachelor of Sciences with a major in life science from National Yang-Ming University in Taipei, Taiwan in 2003, he started a master's degree program in microbiology at National Taiwan University and completed it in 2005. He continued to compulsory military service as a second lieutenant for the next 18 months. After military service in 2007, he worked as a research assistant in the Department of Microbiology at College of Medicine, National Taiwan University for 3 years. In 2010, he entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at Houston.